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(54) Title: INHIBITION OF VIRAL PATHOGENESIS

(57) Abstract: The invention features methods of inhibiting activation of cells by viral erb-B ligands and methods of enhancing immune responses in animals (e.g., human subjects) infected, that will be infected, or are at risk of being infected with a virus whose genome contains a nucleic acid sequence encoding a viral erb-B ligand.



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## Inhibition of Viral Pathogenesis

### TECHNICAL FIELD

This invention relates to control of viral infection, and more particularly to control of poxvirus (e.g., smallpox) infection.

### BACKGROUND

5 In view of the human, economic, and social devastation caused by viral diseases such as hepatitis B and the horrific potential for bioterrorist exploitation of diseases such as smallpox, it is essential that efficacious therapeutic and/or prophylactic agents against such infectious diseases be developed. Because smallpox was considered more than 20 years ago to have been eradicated from the human race, vaccination against the causative virus (variola) ceased at that  
10 time. Thus a relatively high proportion of the present human population has never been vaccinated against the disease. Moreover, only a limited supply of smallpox vaccine produced prior to cessation of vaccination is currently available.

### SUMMARY

15 The inventors have discovered that compounds that inhibit erb-B protein tyrosine kinases are effective at inhibiting poxvirus infections, and thus these compounds are likely to be effective anti-viral therapeutic and prophylactic agents.

Example 2 describes poxvirus proteins with relatively high homology to the mammalian protein epiregulin (EPI), especially in their epidermal factor (EGF)-like domains. These poxvirus proteins are designated herein epiregulin-like growth factors (ELGF). The smallpox  
20 (variola) virus ELGF is designated smallpox growth factor (SPGF) and the vaccinia ELGF is designated vaccinia growth factor (VGF).

The invention is based in part on the observations that binding of the recombinant epidermal growth factor (EGF)-like domain of D4R (the SPGF from the Bangladesh strain of variola major) to cell-surface erb-B1 receptors results in: (a) activation of the tyrosine kinase  
25 activity of such receptors in cells expressing them; (b) enhanced proliferation and/or survival of such cells; (c) rapid internalization of erb-B1 receptors; and (d) viral pathogenesis resulting from enhanced replication and attendant inflammation. These findings indicate that a SPGF (e.g., on

the surface of a variola virion or shed from a variola virion) plays a central role in variola virus entry into, and replication in, host cells and subsequent inflammation.

In addition, the inventors have found that protein tyrosine kinase activity stimulated by binding of the SPGF to cells via the erb-B1 receptor and internalization of the erb-B1 receptor subsequent to ligation by the SPGF are inhibited by erb-B protein tyrosine kinase inhibitors. This inhibition of erb-B1 internalization was apparently due to inhibition of the inducible association between c-Cbl and erb-B1. Consistent with these findings, the inventors also observed that a quinazoline protein tyrosine kinase inhibitor, CI-1033, inhibited clinical symptoms and decreased viral load in animals infected with vaccinia virus. This therapeutic effect of CI-1033 was enhanced by co-administration of a neutralizing vaccinia-specific monoclonal antibody (mAb) (anti-L1R mAb). In addition, the inventors have found that an erb-B tyrosine kinase inhibitor decreased transmission of variola virus from one cell to another.

Interestingly, it was found that CI-1033 also enhanced T cell-mediated immunity to vaccinia virus in the infected animals and that this enhancement was further increased by co-administering the anti-L1R mAb to the infected animals. Moreover, the inventors found that, like CI-1033, a mAb that binds to VGF (the vaccinia ortholog of SPGF) enhanced T cell immunity to vaccinia virus in vaccinia-infected animals and that this effect was further increased by administration of the anti-L1R mAb to the animals.

These findings indicate that inhibitors of erb-B tyrosine protein kinase activity, or inhibitors of activation of protein kinase activity, can be effective agents against poxvirus infection of animals (e.g., human subjects). These inhibitors appear to act by inhibiting, at least, viral replication in cells and viral egress from cells as well as by enhancement of immunity to the virus. The inhibitors may also reduce viral entry into cells.

More specifically, the invention features a method that includes: (a) identifying an animal as likely to have been, or as likely to be, exposed to a virus, the virus containing a gene encoding a viral erb-B ligand; and (b) treating the animal with a compound that inhibits the activity of an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase. The animal can be identified as having been infected, before, during or after step (b), with the virus. The viral erb-B ligand can be a poxvirus erb-B ligand such as an orthopox virus (e.g., variola major, variola minor, monkeypox virus, or vaccinia) erb-B ligand. The erb-B ligand can be an epiregulin-like

growth factor (ELGF) such as smallpox growth factor (SPGF) or vaccinia growth factor (VGF) and the erb-B tyrosine kinase can be erb-B1, erb-B2, or erb-B4 tyrosine kinase.

The compound can be, for example, a non-agonist antibody that binds to the erb-B tyrosine kinase or a non-agonist erb-B ligand or a non-agonist fragment of an erb-B ligand.

Alternatively, the compound can be a small molecule erb-B tyrosine kinase inhibitor such as, for example, a quinazoline-based compound (e.g., 4-anilinoquinazoline), a pyridopyrimidine-based compound, a quinoline-3-carbonitrile-based compound, or a pyrrolopyrimidine-based compound. The small molecule erb-B tyrosine kinase inhibitor can be an irreversible or a reversible inhibitor of the erb-B tyrosine kinase activity. A quinazoline-based compound can be, for example, CI-1033, PD168393, PD160678, PD160879, PD174265, PD153035, ZD1839, GW572016, GW974, OSI-774, or AG1478. A pyridopyrimidine-based compound can be, e.g., PD69896, PD153717, or PD158780, a quinoline-3-carbonitrile-based compound can be, e.g., EKB-569, and a pyrrolopyrimidine-based compound can be, e.g., CGP59326A.

In this method, the animal can be a human. The method can further comprise administering to the animal an antibody that has the ability to substantially neutralize one or more forms of the virus, e.g., an antibody that binds to the intracellular mature virion (IMV) form, the extracellular enveloped virus (EEV) form, and/or the cell-associated envelope virus form (CEV) of an orthopox virus (e.g., variola major, variola minor, vaccinia, or monkeypox virus).

In the method, treatment with the compound can enhance an immune response to the virus in the animal. The immune response can be a T cell response, e.g., a CD8+ T cell response or a CD4+ T cell response, and the T cell response can be an interferon- $\gamma$  (IFN- $\gamma$ )-producing T cell response. Alternatively, the immune response can be an antibody-producing B cell response.

In one embodiment of the method, the virus can be a poxvirus expression vector and can further contain: (a) a heterologous nucleic acid sequence encoding an immunogen; and (b) a transcriptional regulatory element (TRE), the TRE being operably linked to the heterologous nucleic acid sequence. The poxvirus expression vector can be a vaccinia virus expression vector, an attenuated vaccinia virus expression vector, a canarypox virus vector, or a fowlpox virus vector.

The invention also features a method that includes: (a) identifying an animal susceptible to infection by a virus that comprises a gene encoding an erb-B ligand; and (b) treating the animal with (i) an antibody that substantially neutralizes one or more forms of the virus or a vaccine that stimulates an immune response against the virus and (ii) a compound that inhibits an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase. The virus, the animal, the erb-B ligand, the compound, and the antibody that substantially neutralizes one or more forms of the virus can be any of those described above. Moreover, the method can enhance any of the immune responses recited above and the virus can be any of the poxvirus expression vectors described above.

Another aspect of the invention is an *in vitro* method that includes: (a) providing an isolated compound that binds to an erb-B tyrosine kinase and inhibits the activity of an erb-B tyrosine kinase or activation of an erb-B tyrosine kinase; (b) contacting the compound with a cell that expresses the erb-B tyrosine kinase; and (c) before, simultaneous with, or after step (b), contacting the cell with a viral erb-B ligand or a functional fragment of the ligand. The compound can reduce activation of the erb-B tyrosine kinase on the cell by the ligand or fragment. Alternatively, the method can further include determining whether the compound reduces activation of the erb-B tyrosine kinase on the cell by the ligand or fragment. The viral erb-B ligand, the erb-B tyrosine kinase, and the compound can be any of those described above.

The invention also provides a method of determining whether a compound is an antiviral compound. The method includes: (a) providing a compound that inhibits the activity of an erb-B tyrosine kinase or inhibits activation of an erb-B tyrosine kinase; (b) administering the compound to an animal susceptible to infection with a virus that contains a gene encoding an erb-B ligand; (c) before, during, or after step (b), exposing the animal to the virus; and (d) determining whether the compound reduces a symptom of viral infection in the animal. The virus can be a poxvirus, e.g., an orthopoxvirus such as variola major, variola minor, monkeypox virus, or vaccinia. The animal, the erb-B ligand, and the compound can be any of those described above.

Yet another method featured by the invention involves: (a) identifying an animal susceptible to infection by a virus that comprises a gene encoding an erb-B ligand; and (b) treating the animal with an (i) antibody that binds to the ligand and (ii) an antibody that substantially neutralizes one or more forms of the virus or a vaccine that stimulates an immune

response against the virus. The animal can be identified as having been infected, before, during or after step (b), with the virus. The treatment can enhance an immune response to the virus and the immune response can be any of those listed above. The virus can be a poxvirus expression vector and can further contain: (a) a heterologous nucleic acid sequence encoding an immunogen; and (b) a transcriptional regulatory element (TRE), the TRE being operably linked to the heterologous nucleic acid sequence. The poxvirus expression vector can be a vaccinia virus expression vector, an attenuated vaccinia virus expression vector, a canarypox virus vector, or a fowlpox virus vector. The antibody that binds to the ligand can be the 13E8 monoclonal antibody (ATCC accession no. PTA-5040) or the 11D7 monoclonal antibody (ATCC accession no. PTA-5039). The virus, the animal, the erb-B ligand, the compound, and the antibody that substantially neutralizes one or more forms of the virus can be any of those described above.

"Polypeptide" and "protein" are used interchangeably and mean any peptide-linked chain of amino acids, regardless of length or post-translational modification. Polypeptides for use in the invention include those with conservative substitutions. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine, glutamine, serine and threonine; lysine, histidine and arginine; and phenylalanine and tyrosine. In general, variant polypeptides with conservative substitutions will contain no more than 40 (e.g., no more than: 35; 30; 25; 20; 15; 13; 11; 10; nine; eight; seven; six; five; four; three; two; or one) conservative substitution(s). All that is required is that the variant polypeptide have at least 20% (e.g., at least: 30%; 40%; 50%; 60%; 70%; 80%; 90%; 95%; 98%; 99%; 99.5%; 99.8%; 99.9%; or 100% or more) of the activity of the wild-type polypeptide.

As used herein, "activation of a cell" means triggering enhanced proliferation of the cell or enhanced survival of the cell. "Enhanced proliferation of the cell or enhanced survival of the cell" means increased relative to: (a) a lower level of cell proliferation or cell survival; or (b) substantially no cell proliferation or cell survival.

As used herein, "enhancing an immune response" means increasing an immune response relative to: (a) a lower immune response; or (b) substantially no immune response.

The term "isolated compound" as used herein refers to a compound (e.g., a protein) that either has no naturally-occurring counterpart or has been separated or purified from components which naturally accompany it, e.g., in tissues such as pancreas, liver, spleen, ovary, testis,

muscle, joint tissue, neural tissue, gastrointestinal tissue or tumor tissue, or body fluids such as blood, serum, or urine. Typically, a naturally occurring biological compound is considered "isolated" when it is at least 70%, by dry weight, free from proteins and other naturally-occurring organic molecules with which it is naturally associated. Preferably, a preparation of a compound for use in the invention is at least 80%, more preferably at least 90%, and most preferably at least 99%, by dry weight, that compound. The degree of isolation or purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC (high pressure liquid chromatography) analysis. Since a compound (e.g., a protein) that is chemically synthesized is, by its nature, separated from the components that naturally accompany it, the synthetic compound is by definition "isolated."

Isolated compounds, and additional agents useful for the invention, can be obtained, for example, by: (i) extraction from a natural source (e.g., from tissues or bodily fluids); (ii) where the compound is a protein, by expression of a recombinant nucleic acid encoding the protein; or (iii) by standard chemical synthetic methods known to those in the art. A protein that is produced in a cellular system different from the source from which it naturally originates is "isolated," because it will necessarily be free of components which naturally accompany it.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

Other features and advantages of the invention, e.g., inhibiting activation of cells, will be apparent from the following description, from the drawings and from the claims.

## DESCRIPTION OF DRAWINGS

Fig. 1 is a photograph of a Coomassie blue-stained SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel of recombinant forms of the epidermal growth factor (EGF)-like domains of murine epiregulin ("Epi"), human EGF, and variola major Bangladesh strain smallpox growth factor ("SPGF"). These recombinant proteins are referred to herein as recEGF, recEPI, and recSPGF, respectively. The positions on the gel of molecular weight (MW) markers are indicated on the left side of the photograph, the values shown being MW x 10<sup>-3</sup>.

Fig. 2 is a depiction of amino acid sequence alignments (for optimum homology) of human epiregulin (SEQ ID NO:6) and mouse epiregulin (SEQ ID NO:7) and the orthopox viral orthologs D1L (SEQ ID NO:1), CMP11R (SEQ ID NO:3), C11R (SEQ ID NO:4), and D3R (SEQ ID NO:5) from variola major strain India, camelpox, vaccinia, and monkeypox viruses, respectively. The regions corresponding to leader sequences and transmembrane domains are indicated and the most conserved segments of the EGF-like domains are boxed and labeled "EGF".

Figs. 3A and 3B are depictions of the amino acid sequences of the SPGF of variola major strain India (D1L) (SEQ ID NO:1) and variola major strain Bangladesh (D4R) (SEQ ID NO:2), respectively. Positions at which the two sequences differ are shown in bold face and underlined.

Fig. 4 is a line graph showing the percentages of human primary fibroblasts (SC-J) in the S-phase of the cell cycle after 18 hours of culture in the indicated concentrations of recEGF ("EGF"), recEPI ("EPI"), and recSPGF ("SPGF").

Fig. 5 is bar graph showing the proliferation of primary human keratinocytes ("N keratinocyte") and primary human fibroblasts ("R2F fibroblast") during culture in either standard tissue culture medium containing 10% fetal bovine serum (FBS) ("Complete") or in tissue culture medium containing 2% FBS and the indicated concentrations of recSPGF ("SPGF") or recEGF ("EGF").

Fig. 6 is a pair of photographs of western blots of immunoprecipitates from MB453 cells ("453") or MB468 cells ("468"). The immunoprecipitates were prepared by incubating the cells with biotinylated recSPGF, exposing the cells to the cross-linking agent BS<sup>3</sup> (in order to cross-link biotinylated recSPGF molecules bound to erb-B receptors on the surface of the cells), lysing the cells, and immunoprecipitating ("IP") aliquots of the lysates with antibodies specific for the four erb-B receptors, i.e., erb-B1 ("B1"), erb-B2 ("B2"), erb-B3 ("B3"), or erb-B4 ("B4"). Two duplicate western blots were generated from the immunoprecipitates; one was stained with horseradish peroxidase-conjugated streptavidin ("Sav-HRPO") (Fig. 6, upper panel) and the other was stained with each of the erb-B receptor-specific antibodies (Fig. 6, middle panel). The bottom panel of Fig. 6 shows the expression of the four erb-B receptors by MB453 ("453") and MB468 ("468") cells.

Fig. 7 is a line graph showing the binding to MB468 cells ("on 468") of the indicated amounts ("ng") of biotinylated recSPGF (detected with phycoerythrin (PE)-conjugated streptavidin) ("SPGF") in the absence (open circles) and presence (" +mAb"; closed circles) of a blocking antibody specific for erb-B1. The data are presented as mean fluorescence intensities ("MFI"), which were determined by fluorescence flow cytometry (FFC). Binding of the recSPGF to MB453 cells ("on 453"), which do not express erb-B1, was also tested ("on 453"; open triangles).

Fig. 8 is a line graph showing the relative ability (expressed as % inhibition) of the indicated concentrations of recSPGF ("SPGF"), recEPI ("EPI"), and recEGF ("EGF") to inhibit the binding of biotinylated recSPGF to MB468 cells.

Fig. 9 is a Scatchard plot of recSPGF binding to MB468 cells. The analysis indicated the presence on the cells of approximately  $5.5 \times 10^4$  high affinity receptors (" $K_D = 0.14$  nM") and approximately  $8.8 \times 10^6$  low affinity receptors (" $K_D = 258$  nM") for SPGF on the cells.

Figs. 10A-D are a series of fluorescence photomicrographs showing the cellular distribution of erb-B1 receptors (as detected with a mouse antibody specific for erb-B1 receptors and fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig antibody) on HeLa cells that

either were unstimulated (“(-)”; Fig. 1A) or were stimulated for 10 minutes with either recSPGF (“SPGF”; Fig. 10B), recEPI (“EPI”; Fig. 10C), or recEGF (“EGF”; Fig. 10D).

Fig. 11A is a photograph of a western blot of whole cell lysates of HeLa cells that were either untreated (“(-)”) or were stimulated with recSPGF (“SPGF”), recEPI (“EPI”), or recEGF (“EGF”). The western blot was stained with a mAb specific for phosphotyrosine residues (“WB: 4G10”). The positions on the gel of molecular weight (MW) markers are shown on the left of the photograph, the values shown being MW x 10<sup>-3</sup>.

Fig. 11B is a photograph of a western blot of immunoprecipitates of cell lysates of HeLa cells that were either untreated (“(-)”) or were stimulated with recSPGF (“SPGF”), recEPI (“EPI”), or recEGF (“EGF”). The immunoprecipitates were prepared with an antibody specific for erb-B1 (“IP: EGFR”). The western blot was stained with a mAb specific for phosphotyrosine residues (“WB: 4G10”).

Figs. 12A-12D are depictions of: the generic chemical structure of 4-anilinoquinazoline compounds (“Generic 4-anilinoquinazoline”; Figure 1D) showing the numbering of relevant atoms; and three specific 4-anilinoquinazoline compounds, PD153035 (Fig. 12A), PD168393 (Fig. 12B), and CI-1033 (Fig. 12 C).

Fig. 13 is a photograph of a western blot of whole cell lysates of HeLa cells that were untreated (“(-)”) or were stimulated with recSPGF (“SPGF”) either without pre-exposure to an erb-B protein tyrosine kinase inhibitor (“(+)”) or with pre-exposure to one of three erb-B protein tyrosine kinase inhibitors, PD153035, PD158780, and PD168393. The western blot was stained with a mAb specific for phosphotyrosine residues. The positions on the gel of molecular weight (MW) markers are shown on the left of the photograph, the values shown being MW x 10<sup>-3</sup>.

Fig. 14A is a line graph showing the percentages of human primary fibroblasts (SC-J) in the S-phase of the cell cycle after: no pretreatment (“SPGF alone”) or pretreatment with either PD153035, CI-1033, or PS168393; and stimulation overnight with the indicated concentrations of recSPGF (“ng/ml”).

Fig. 14B is a photograph of a western blot of whole cell lysates of HeLa cells that were either untreated (“(-)”) or were stimulated with recSPGF (“SPGF”) without pre-exposure to an erb-B protein tyrosine kinase inhibitor (“(+)”) or with pre-exposure to the Src family protein tyrosine kinase inhibitor PP2 or the the erb-B protein tyrosine kinase inhibitors PD153035, PD168393 or CI-1033. The western blot was stained with a mAb specific for phosphotyrosine residues (“WB: P-Y (4G10)”). The positions on the gel of molecular weight (MW) markers are shown on the left of the photograph, the values shown being MW x 10<sup>-3</sup>. The arrows on the right side of the photograph indicate the positions of proteins phosphorylation of which was affected by erb-B1 protein tyrosine kinase inhibition.

Fig. 14C is a bar graph showing the level of expression (in MFI determined by FFC) of erb-B1 receptors on A431 cells after: pretreatment with no inhibitor or pretreatment with PP2, PD153035, PD168393 or CI-1033; and a 5 minute incubation with biotinylated recSPGF.

Fig. 14D is a series of photographs of western blots of immunoprecipitates (“IP”) of cell lysates of HeLa cells that were either untreated (“(+)”) or pretreated with PP2, PD153035, PD168393 or CI-1033 and then stimulated for 5 minutes with recSPGF. A control in which the cells were neither pretreated nor stimulated recSPGF (“(-)”) was also performed. The immunoprecipitates (“IP”) were prepared with either an antibody specific for erb-B1 (“EGFR”) or an antibody specific for c-Cbl (“c-Cbl”). The western blots were stained (“WB”) with mAbs specific for erb-B1 (“EGFR”; upper panel) or c-Cbl (middle and lower panel).

Fig. 15A is a line graph showing the survival (in percentages of mice in the various treatment groups) over time of mice that were challenged intranasally with vaccinia virus and either untreated (“Control”; open circles), pretreated once with anti-L1R mAb (“anti-L1R”; closed circles), treated daily with CI-1033 (“CI-1033”; open triangles), or pretreated once with anti-L1R mAb and treated daily with CI-1033 (“CI-1033 + anti-L1R”; closed inverted triangles).

Fig. 15B is a line graph showing the clinical score (derived as described in Example 1) of the mouse experimental groups described for Fig. 15A. The same symbols used in Fig. 15A are used to indicate the various experimental groups.

5 Fig. 15C is a bar graph showing the average weight of a whole lung from mice six days after a lethal challenge with vaccinia virus and either no treatment ("Control"), pretreatment with anti-L1R mAb ("anti-L1R"), daily treatment with CI-1033 ("CI-1033"), or one pretreatment with anti-L1R mAb and daily treatment with CI-1033 ("CI-1033 + anti-L1R").

10 Fig. 15D is a bar graph showing the average viral titer (in "Log<sub>10</sub>PFU"; PFU, plaque forming units) in the lungs of mice eight days after infection with vaccinia virus and either one pretreatment with anti-L1R mAb ("anti-L1R"), daily treatment with CI-1033 ("CI-1033"), one pretreatment with the 13E8 mAb, one pretreatment with anti-L1R mAb and the 13E8 mAb, or one pretreatment with anti-L1R mAb and daily treatment with CI-1033 ("CI-1033 + anti-L1R").  
15 The control group ("None"; hatched bar), in which mice were infected with virus but received no pretreatment or treatment, was from a separate experiment in which the mice were sacrificed on day 7 since all in the mice in the control group in the present experiment were dead on day 8.

Fig. 16A is a photograph of an autoradiogram from an Rnase protection assay using RNA  
20 prepared from the lungs of mice: that were either uninfected ("0"); on the indicated days ("4", "6", or "8") after infection with vaccinia virus. Infected animals were either untreated ("Control"), pretreated once with anti-L1R mAb ("anti-L1R"), treated daily with CI-1033 ("CI-1033"), or pretreated once with anti-L1R mAb and treated daily with CI-1033 ("CI-1033 + anti-L1R"). The lung RNA was hybridized with the cytokine-specific probes in the mCK-2b  
25 template set (BD Biosciences-Pharmingen, San Diego, CA) as indicated on the left side of the photograph. Protected fragments were resolved on a DNA sequencing gel, which was then exposed to x-ray film. Cytokine mRNA whose levels were increased by CI-1033 (alone or in combination with anti-L1R mAb) are indicated by arrows on the right side of the photograph.

30 Fig. 16B is a series of two-dimensional FFC profiles showing the levels of intracellular interferon- $\gamma$  ("IFN- $\gamma$  (log)") in, and levels of cell surface CD44 ("CD44 (log)") on, mouse

spleen CD8<sup>+</sup> cells that had been stimulated *in vitro* with: normal MC57G cells ("MC57G"); or vaccinia virus-infected MC57G cells ("VV-infected MC57G"). The experimental groups consisted of mice that had been infected with vaccinia virus (5 days prior to sacrifice and assay) and were untreated ("Control"), pretreated once with anti-L1R mAb ("anti-L1R"), treated daily with CI-1033 ("CI-1033"), or pretreated once with anti-L1R mAb and treated daily with CI-1033 ("CI-1033 + anti-L1R"). The data shown are from a representative mouse from each experimental group of mice.

Fig. 17 is a series of two-dimensional FFC profiles showing the levels of intracellular interferon- $\gamma$  ("IFN- $\gamma$  (log)") in, and levels of cell surface CD44 ("CD44 (log)") on, mouse spleen CD8<sup>+</sup> cells that had been stimulated *in vitro* with normal MC57G cells ("MC57G"); vaccinia virus-infected MC57G cells ("VV-infected MC57G"); or anti-CD3 mAb ("anti-CD3"). The experimental groups consisted of mice that were uninfected ("Naïve") or mice that had been infected with vaccinia virus (5 days prior to sacrifice and assay) and were untreated ("Control"); pretreated once with anti-L1R mAb ("anti-L1R"); pretreated once with the 13E8 mAb ("13E8"), or pretreated once with anti-L1R mAb and the 13E8 mAb ("anti-L1R + 13E8"). The data shown are from a representative mouse from each experimental group of mice.

Fig. 18A is a series of photographs showing the immunohistochemical staining of variola strain Solaimen plaques obtained by culturing BSC-40 cell monolayers in either the absence ("-") or presence ("+") of the indicated concentrations of CI-1033.

Fig. 18B is a bar graph showing the effect of CI-1033 on the number of plaques obtained by culturing BSC-40 cell monolayers in either the absence ("0") or presence of the indicated concentrations of CI-1033.

Fig. 18C is a bar graph showing the number of comets obtained by culturing BSC-40 cell monolayers in either the absence ("0") or presence of the indicated concentrations of CI-1033.

## DETAILED DESCRIPTION

The experimental findings outlined above in the Summary section indicate that compounds that inhibit either erb-B1 protein tyrosine kinase activity or the stimulation of erb-B1 protein tyrosine kinase activity can be effective in treatment of, and/or prophylaxis against, orthopox virus-mediated pathogenesis (e.g., smallpox or vaccinia infection). Although the invention is not limited by any particular mechanism of action, it is believed that such compounds may act by inhibiting entry of virus particles into a host cell, intracellular viral replication, and/or expulsion of the virus from the cell, and by enhancing adaptive T cell immunity to the viruses. In that the genomes of orthopox viruses contain genes that encode immunosuppressive factors, it is very possible that the inhibition of viral replication and enhancement of adaptive immunity are functionally related. Moreover, previous findings showing roles for erbB receptors in the infection of cells by, for example, hepatitis B virus, Epstein-Barr virus, RNA tumor viruses (e.g., avian erythroblastosis virus), cytomegalovirus (CMV), and reovirus (see references in Example 7), in light of the present observations, suggest that inhibitors of erb-B protein tyrosine kinases are likely to be effective therapeutic and/or prophylactic agents against not only multiple poxviruses but also a wide variety of other viruses.

Of particular interest are the smallpox (variola major and variola minor) and cowpox (vaccinia) viruses. These viruses are large double-stranded DNA viruses that replicate in the cytoplasm of infected cells. Variola virus enters subjects through the respiratory tract; human-human transmission usually occurs as a result of coughing out of virus in oralpharyngeal secretions. The incubation period is 7-19 days, followed by fever, headache and backache. After 2-3 days, the fever falls and a rash appears on the face, trunk, and extremities and progresses to vesicles, pustules and scabs lasting for several weeks. Variola major infection is fatal in approximately 40% of unvaccinated human beings. Death is primarily due to internal bleeding (disseminated intravascular coagulation) and vascular collapse. Infection with variola minor results in a less severe form of smallpox with reduced fatality.

Since international eradication of smallpox in about 1980, vaccination has ceased. Thus, smallpox protection has not been provided in the United States for approximately 20 years. As a result, a large proportion of the population has never been vaccinated against smallpox. Moreover, there are presently inadequate reserves of smallpox vaccine (attenuated vaccinia virus) in the United States, and what is available was produced by a poorly controlled process

which yielded live vaccinia virus capable of adversely affecting immunodeficient (e.g., AIDS and cancer) patients. In addition, 10-20 second generation cases can result from a single human infection with variola. These factors make variola virus, and particular variola major virus, an obvious choice as a bioweapon against the population of the United States. Thus it is imperative that efficient, safe therapeutic and/or prophylactic methodologies against the virus be developed as quickly as possible.

Variola is one of the largest and most complex of double-stranded DNA viruses and is visible by light microscopy. Sequence data are available on variola major viruses and variola minor virus. Current sequence data for variola in GenBank include: (a) five nucleotide sequence entries and 626 amino acid sequence entries for variola major; and (b) three nucleotide sequence entries and 619 amino acid sequence entries for variola minor. There are two complete variola major genome nucleotide sequences, corresponding to strain India 1967 (GI:9627521; 185,578 bp) and strain Bangladesh (GI:623595; 186,103 bp), and one complete genome nucleotide sequence of variola minor strain Alastrim (GI:5830555; 186,986 bp) available in GenBank. Comparison of the variola major and variola minor genomes shows that they are over 97% identical throughout the entire genome. Likewise, comparison of any of the variola genomes with that of vaccinia (GI:9790357; 191,737 bp) indicates that they are ~95% identical.

There are 197 genes in the genome of variola major strain India 1967 (GI:9627521). Very little is known about their function and relevance in the life cycle of the virus. Data from a computer-based analysis of the genome of a variola virus (variola major strain India) identified a variety of polypeptides encoded by genes in the genome that are potentially useful as vaccines against smallpox and for generating antibodies that would be effective passive immunoprotectants (see co-pending U.S. Application Serial No. 10/429,685 whose disclosure is incorporated herein by reference in its entirety). One of the polypeptides identified by this analysis was D1L, which is an epidermal growth factor (EGF)-like membrane-bound protein with  $\geq 97\%$  homology to corresponding molecules of other variola strains, 86-89% identity with the corresponding vaccinia protein, and 30% identity to human epiregulin. As indicated above, these poxvirus EGF-like membrane-bound proteins are referred to herein collectively as epiregulin-like growth factors (ELGF); ELGF from variola strains are referred to herein as smallpox growth factors (SPGF) and those from vaccinia strains as vaccinia growth factors (VGF). In Figs. 3A and B are shown the amino acid sequences of SPGF from two variola

strains, i.e., D1L from variola major India (Fig. 3A; SEQ ID NO:1) and D4R from variola major Bangladesh (Fig. 3B; SEQ ID NO:2). Note that these two SPGF differ at only two amino acid positions (indicated in bold and underlined in Figs. 3A and B).

Various aspects of the invention are described below.

#### Methods of Inhibiting Activation of a Cell

The invention includes methods of inhibiting activation of a cell that expresses a cell surface erb-B protein tyrosine kinase molecule. In these methods, one or more isolated compounds that inhibit erbB protein tyrosine kinase activity or inhibit the activation of erb-B protein tyrosine kinase activity are delivered to the cell of interest. Delivery to the cell of the one or more compounds can occur prior to, simultaneous with, or after contacting of the cell with a viral erb-B ligand (e.g., an ELGF) or a functional (i.e., erb-B-binding and activating) fragment of an erb-B ligand. The method can include, prior to the delivery step, identifying a cell expressing an erb-B protein tyrosine kinase. The cell can be any cell expressing an erb-B protein tyrosine kinase molecule, e.g.: any kind of epithelial cell, for example, a pulmonary epithelial cell such as a lung epithelial cell, a gastrointestinal epithelial cell (e.g. a stomach or colonic epithelial cell), a skin epithelial cell (e.g., a keratinocyte), or an epithelial cell of the genitourinary system (e.g., a bladder or uterine epithelial cell); or a fibroblast. The cells can be normal cells or malignant cells. The erbB protein tyrosine kinase can be erb-B1, erb-B2, or erb-B4 protein tyrosine kinase, a homodimer of any of these three proteins, or a heterodimer between any two of erb-B1, erb-B2, erb-B3, and erb-B4 protein tyrosine kinases.

The viral erb-B ligand (EBL) can be from any virus whose genome contains a nucleic acid (DNA or RNA) encoding a protein that binds to an erb-B protein tyrosine kinase. The viral EBL can be, for example, from any of the viruses listed herein. It can be, e.g., from poxviruses. Poxviruses (family Poxviridae) of interest include but are not limited to Orthopoxviruses (e.g., variola, vaccinia, and monkeypox), Avipoxviruses (e.g., fowlpox and canarypox), Capripoxviruses (e.g., sheep poxvirus), Leporipoxviruses (e.g., myxoma and Shope virus), Parapoxviruses (e.g., orf virus and swinepox), Molluscipoxviruses (e.g., Molluscum contagiosum virus), and Yatapoxviruses (e.g., Yata monkey tumor virus). Of particular interest are variola (variola major and variola minor) and vaccinia viruses. The poxvirus ELGF can be, for example, a SPGF (e.g., the D1L protein from the variola major India strain or the D4R protein

from the variola major Bangladesh strain) or a VGF (from vaccinia virus). A functional fragment of a viral EBL, as used herein, is a fragment that is shorter than the full-length, wild-type, mature protein but has at least 20% (e.g., at least: 30%; 40%; 50%; 60%; 70%; 80%; 90%; 95%; 97%; 98%; 99%; 99.5%; 99.7%; 99.8%; 99.9%, or 100% or more) of the ability of the full-length, wild-type, mature protein to bind to one or more of the erb-B receptors to which the full-length, wild-type protein binds. Methods of measuring the ability of ligands to bind to receptors are known in the art (see below for an example of such a method). Functional fragments can, for example, be or contain an EGF-like domain, e.g., amino acids 40 to 90 of SPGF D1L (SEQ ID NO:1) or D4R (SEQ ID NO:2). By sequence alignment (see Fig.2), those skilled in the art will be able to discern which regions of an EBL of interest contain or are an EGF-like domain.

The viral EBL that contacts the cell-surface erb-B protein tyrosine kinase can be a component of a viral particle, and indeed binding of a viral EBL in this form to a cell-surface erb-B molecule can be at least one early step in the infection of the cell by the viral particle. Alternatively, the viral EBL (or a functional fragment thereof) can either be secreted by a cell infected with the virus or be shed from an extracellular viral particle. In addition, the viral EBL (or functional fragment thereof) can be a molecule that has been, for example, partially or completely purified from the virus or has been chemically or recombinantly produced.

A compound that inhibits an erb-B protein tyrosine kinase can be an antibody that binds to an erb-B protein. Such an antibody can be a non-agonist antibody, i.e., an antibody that activates substantially no signal through the receptor to which it binds. Antibodies specific for the ectodomain of erb-B1 have been described that compete for ligand binding and block erb-B1 signaling; moreover these antibodies were shown to cause cell cycle arrest and/or cell death [Arteaga (2001) J. Clin Oncol. 19(18) (September 15 supplement): 32s-40s and references cited therein].

Another class of relevant compounds includes antibodies that bind to a viral EBL, e.g., a mAb that binds to an SPGF or VGF such as the 3D4R-13E8 and 3D4R-11D7 mAbs (also referred to herein as the 13E8 and 11D7 mAbs, respectively).

Moreover, a compound useful for the method of the invention can be a non-agonist erb-B ligand or a non-agonist fragment of an erb-B ligand that binds to an erb-B protein. Methods for determining whether a compound that binds to a receptor of interest is an agonist or a non-agonist of that receptor are known in the art. Mammalian ligands of erb-B include, but are not

limited to, EGF, epiregulin, transforming growth factor- $\alpha$  (TGF $\alpha$ ), amphiregulin, heparin binding EGF, betacellulin, and heregulin [Arteaga et al. (2001) J. Clin. Oncol. 19:32s-40s]. Other appropriate compounds include non-agonist fragments of viral proteins, e.g., an ELGF such as SPGF or a VGF.

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Small molecule erb-B tyrosine kinase inhibitors

Compounds of particular interest are small molecule inhibitors of erb-B protein tyrosine kinase activity. A large number of such compounds have been described. There are essentially three categories of such compounds. In category A are compounds that appear to act substantially by competing for the Mg-ATP binding site of the catalytic domain of erb-B protein tyrosine kinases [Al-Abeidi (2000) Oncogene 18:5690-5701]. In category B are compounds that apparently act substantially by competing for the tyrosine site on the erb-B protein tyrosine kinase [Posner et al. (1994) Mol. Pharmacol. 45:673-683, incorporated herein by reference in its entirety]. In category C are compounds that appear to act by both mechanisms [Posner et al. (1994) Mol. Pharmacol. 45:673-683].

Category A compounds include, but are not limited to, bicyclic quinazoline-based compounds (e.g., 4-anilinoquinazolines), pyridopyrimidine-based compounds, quinoline-3-carbonitrile-based compounds, pyrrolopyrimidine-based compounds, pyrazolopyrimidine-based compounds, as well as tricyclic derivatives of quinazolines (such as imidazoquinazolines, pyrroloquinazolines, and pyrazoloquinazolines) and the other bicyclic compounds listed above [Fry et al. (2003) Exp. Cell Res. 284:131-139, incorporated herein by reference in its entirety].

Examples of quinazoline-based compounds include: ZD1839/Iressa [Fry et al. (2003)]; GW572016 (N-{3-Chloro-4-[3-fluorobenzyl]oxy}phenyl}-6-[5-({2-methylsulfonyl}ethyl)amino}methyl)-2-furyl]-4-quinazoline [Xia et al. (2002) Oncogene 21:6255-6263; and Cockerill et al. (2001) Bioorg. Med. Chem. Lett. 11:1401-1405, both incorporated herein by reference in its entirety]; GW974 [Cockerill et al. (2001); and Rusnak et al. (2001) Cancer Res. (2001) 61:7196-7203, incorporated herein by reference in its entirety]; PD168393 [Fry et al. (1998) Proc. Natl. Acad. Sci. USA 95:12022-12027, incorporated herein by reference in its entirety; Fig. 12], PD153035/ZM252868/AG1517 [Fry et al. (1994) Science 265:1093-1095, incorporated herein by reference in its entirety; Fig. 12], and CI-1033 [Pfizer; Fig. 12]; OSI-774/CP-358,774/Tarceva [Fry et al. (2003); and Stamos et al. (2002) J. Biol.

Chem. 277:46265-46272, incorporated herein by reference in its entirety]; AG1478 [Levitzki et al. (1995) Science 267:1782-1788, both incorporated herein by reference in its entirety]; and PD160678, PD160879, PD168393, and PD174265 [Fry et al. (1998) Proc. Natl. Acad. Sci. USA 95:12022-12027].

5           Examples of useful pyridopyrimidine-based compounds include PD69896, PD153717, and PD158780 [Fry et al. (1997) Biochem. Pharmacol. 54(8):877-887, incorporated herein by reference in its entirety]. A useful quinoline-3-carbonitrile is EKB-569 [Fry et al. (2003)], a useful pyrrolopyrimidine is CGP59326A/PKI-166 [Lydon et al. (1998) Int. J. Cancer 76(1):154-163, incorporated herein by reference in its entirety], and useful pyrazolopyrimidines include, for  
10           example, compounds 9 and 11 in Traxler et al. [(1997) J. Med. Chem. 40:3601-3616, incorporated herein by reference in its entirety].

          It is clear that multiple derivatives of all these classes of compounds having a wide range of substituents demonstrate erb-B tyrosine kinase inhibitory activity and are thus likely candidates for the methods of the invention [see, for example, Thompson et al. (1995) J. Med.  
15           Chem. 38:3780-3788; Rewcastle et al. (1996) J. Med. Chem. 39:1823-1835; Rewcastle et al. (1997) J. Med. Chem. 40:1820-1826; Wissner et al. (2000) J. Med. Chem. 43:3244-3256; Traxler et al. (1996) J. Med. Chem. 39:2285-2292; Traxler et al. (1997) 40:3601-3616; Rewcastle et al. (1996) J. Med. Chem. 39:918-928; and Palmer et al. (1997) J. Med. Chem. 40:1519-1529, all of which are incorporated herein by reference in their entirety].

20           Some of the above compounds (e.g., CI-1033, PD168393, EKB-569, PD1606678, PD160879, and PD174265), by virtue of an acrylamide group, act as irreversible inhibitors by alkylating, for example, the Cys<sup>773</sup> residue of erb-B1. A comparison of the relative efficacy of a series of such acrylamide derivatives is described in Smaill et al [(1999) J. Med. Chem. 42(10): 803-1815, incorporated herein by reference in its entirety]. In addition, it has been shown that  
25           substituting these compounds (e.g., at the 7 position of quinazoline and pyridopyrimidine compounds) with alkylamine and alkoxyamine side chains serves to overcome aqueous solvent solubility problems [Smaill et al. (2000) J. Med. Chem. 43(7)1380-1397, incorporated herein by reference in its entirety].

          Also in category A are compound DAPH 1 (4,5-Dianilinophthalimide) and derivatives of  
30           it [Buchdunger et al. (1994) Proc. Natl. Acad. Sci. USA 91:2334-238, incorporated herein by

reference in its entirety] and the fungal products quercetin, genistein and lavendustin A [Levitzki et al. (1995) Science 267:1782-1788, incorporated herein by reference in its entirety].

In category B are the compounds AG537 and AG538 [Posner et al. (1994) Mol. Pharmacol. 45:673-683] and flavone derivatives such as compounds 10a, 10c, and 10m in Cushman et al. [(1994) J. Med. Chem. 37:3353-3362, incorporated herein by reference in its entirety].

In category C are: thiazolidine-diones such as compounds 1, 2, and 3 in Geissler et al. [(1990) J. Biol. Chem. 265(36):22255-22261, incorporated herein by reference in its entirety]; 3-substituted 2,2'-diselenobis(1H-indoles) such as compounds 33 and 34 in Showalter et al. [(1997) J. Med. Chem. 40:413-426, incorporated herein by reference in its entirety]; 2,3-dihydro-2-thioxo-1H-indole-3-alkanoic acids and 2,2'-dihydrobis(1H-indole-3-alkanoic acids) such as compounds 9, 32, and 33 in Thompson et al. [(1993) J. Med. Chem. 36:2459-2469, incorporated herein by reference in its entirety]; and [(alkylamino)methyl]acrylophenones and (alkylamino)propiophenones such as compounds 5, 17, 18, and 19 in Traxler et al. [(1995) J. Med. Chem. 38:2441-2448, incorporated herein by reference in its entirety].

The term "small molecule erb-B tyrosine kinase inhibitors", as used herein, refers to all the compounds described above under the title "*Small molecule erb-B tyrosine kinase inhibitors*".

Both large molecule (e.g., antibodies) and small molecule compounds can be delivered to a cell singly or in combinations of 2 or more, e.g., three, four, five, six, seven, eight, nine, ten, 15, or 20. Such combinations can include combinations involving only one or more than one (e.g., two, three, or four) of the above-described classes of inhibitors. Moreover, supplementary agents can be delivered to the cell or the environment of the cell, e.g., cultures or animals containing the cell, in order to enhance the effect of the above-described compounds. One such supplementary agent that can be used is an antibody that binds to one or more forms of the virus containing a gene that encodes the EBL of interest and substantially neutralizes the virus. As used herein, "substantially neutralizes" means has the ability to reduce the titer of virus at least 5-fold, e.g., at least: 10-fold; 20-fold; 50-fold; 100-fold; 1,000-fold; 10,000-fold; 100,000-fold; 1,000,000-fold or more. Examples of such antibodies include neutralizing antibodies that bind to poxvirus intracellular mature virions (IMV) (e.g., an antibody specific for the L1R vaccinia

protein or for its variola orthologue), extracellular enveloped virus (EEV), or cell-associated enveloped virus (CEV).

In certain *in vivo* embodiments, instead of administering a neutralizing antibody to a subject, the subject can be vaccinated with a source of an appropriate antigen in order to generate a neutralizing antibody *in situ*. Appropriate antigens and methods of immunization are described below in the section on Methods of Enhancing an Immune Response.

All the antibodies described above can be polyclonal antibodies or mAb and can be from any of a wide range of species, e.g., a human, a non-human primate (e.g., a monkey or a chimpanzee), a cow, a horse, a goat, a sheep, a pig, a cat, a dog, a rabbit, a guinea pig, a hamster, a gerbil, a rat, a mouse, or a chicken.

As used herein, the term "antibody" refers not only to whole antibody (e.g., IgM, IgG, IgA, IgD, or IgE) molecules, but also to antigen-binding fragments, e.g., Fab, F(ab')<sub>2</sub>, Fv, and single chain Fv (scFv) fragments. An scFv fragment is a single polypeptide chain that includes both the heavy and light chain variable regions of the antibody from which the scFv is derived. Also included are chimeric antibodies, e.g., humanized antibodies.

Antibody fragments that contain the binding domain of the molecule can be generated by known techniques. For example: F(ab')<sub>2</sub> fragments can be produced by pepsin digestion of antibody molecules; and Fab fragments can be generated by reducing the disulfide bridges of F(ab')<sub>2</sub> fragments or by treating antibody molecules with papain and a reducing agent. See, e.g., National Institutes of Health, 1 Current Protocols In Immunology, Coligan *et al.*, ed. 2.8, 2.10 (Wiley Interscience, 1991). scFv fragments can be produced, for example, as described in U.S. Patent No. 4,642,334, which is incorporated herein by reference in its entirety.

Chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example, using methods described in Robinson *et al.*, International Patent Publication PCT/US86/02269; Akira *et al.*, European Patent Application 184,187; Taniguchi, European Patent Application 171,496; Morrison *et al.*, European Patent Application 173,494; Neuberger *et al.*, PCT Application WO 86/01533; Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent Application 125,023; Better *et al.* (1988) Science 240, 1041-43; Liu *et al.* (1987) J. Immunol. 139, 3521-26; Sun *et al.* (1987) PNAS 84, 214-18; Nishimura *et al.* (1987) Canc. Res. 47, 999-1005; Wood *et al.* (1985) Nature 314, 446-49; Shaw *et al.* (1988) J. Natl. Cancer Inst. 80, 1553-59; Morrison, (1985) Science 229, 1202-07;

Oi et al. (1986) BioTechniques 4, 214; Winter, U.S. Patent No. 5,225,539; Jones et al. (1986) Nature 321, 552-25; Veroeyan et al. (1988) Science 239, 1534; and Beidler et al. (1988) J. Immunol. 141, 4053-60.

5 Fully human antibodies (polyclonal or monoclonal) can be produced by immunizing transgenic animals (e.g., mice) that contain gene segments encoding gene segments encoding all human immunoglobulin (i.e., variable, joining, diversity, and constant) regions (see, for example, U.S. Patent Nos. 5,545,806 and 5,569,825).

10 Applicants have deposited under the Budapest Treaty the 3D4R-11D7 and 3D4R-13E8 hybridomas with the American Type Culture Collection (ATCC), Rockville, MD 20852, U.S.A. The 3D4R-11D7 hybridoma was assigned the ATCC accession no. PTA-5039 and the 3D4R-13E8 hybridoma the ATCC accession no. PTA-5040. The hybridomas deposited with the ATCC were taken from a deposit maintained by the Dana Farber Cancer Institute, Inc., since prior to the priority date of this application. The deposits of hybridomas will be maintained without restriction in the ATCC depository for a period of 30 years, or five years after the most recent request, or for the effective life of the patent, whichever is the longer, and will be replaced if the deposit becomes non-viable during that period.

The methods of inhibiting activation of a cell can be *in vitro* or *in vivo*.

20 *In vitro* application of the methods of the invention can be useful in basic scientific studies on viral infection and mechanisms of cellular resistance to viral infection. In the *in vitro* methods of the invention, the compounds can be cultured with cells of interest (see above) and any of the forms of the viral EBL recited above. Measurements of, for example, viral titer, level of cell proliferation/survival, and/or protein phosphorylation can be made after various times of incubation using methods described herein and those known in the art.

25 Moreover, such methods can be useful for screening test compounds for their ability to inhibit viral infection of cells. In such assays, a test compound binds to an erb-B receptor interest can be contacted with a cell that expresses the erb-B receptor by culturing the test compound with the cell. Before, simultaneous with, or after the contacting, the cell can be contacted with a viral EBL or a functional fragment of an EBL. A determination is then made of whether the test compound reduces activation of the erb-B receptor by the ligand or the functional fragment. Activation of erb-B receptors can be measured by methods known in the art, e.g., by (a) detecting or measuring tyrosine phosphorylation of the erb-B receptor or

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downstream intracellular proteins that are phosphorylated directly by the activated erb-B receptor or indirectly by proteins activated in a cascade- fashion following activation of the erb-B receptor; (b) detecting or measuring internalization of the erb-B receptor; or (c) detecting or measuring proliferation and/or survival of a cell.

5           The *in vitro* methods of the invention in which the compound used is known to activate an erb-B receptor can also be "positive controls" in the above-described screening assays for compounds with the ability to inhibit activation of a cell by a viral EBL.

10           The methods of the invention will preferably be *in vivo*. These applications can be useful in the therapy and prophylaxis of relevant viral diseases. They can also be useful for diminishing the side effects of vaccination with live virus (e.g., cowpox). By administering a compound of interest to an animal infected (e.g., a human smallpox patient), an animal that will be infected (e.g., a human subject to be vaccinated with vaccinia virus), or an animal at risk of being infected (e.g., a human subject at risk of being infected by a variola major virus in the course of an anticipated bioterrorist attack), with an EBL-expressing virus, therapy of or prophylaxis from the  
15           clinical symptoms caused by the virus can be achieved.

20           As used herein, "prophylaxis" can mean complete prevention of the symptoms of a disease, a delay in onset of the symptoms of a disease, or a lessening in the severity of subsequently developed disease symptoms. As used herein, "therapy" can mean a complete abolishment of the symptoms of a disease or a decrease in the severity of the symptoms of the disease.

25           Modifications of the above-described *in vivo* methods of the invention can be used as screening assays for compounds that are effective prophylactic and/or therapeutic agents against a virus of interest. In such a method, a test compound that inhibits the activity of an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase is administered to an animal that is susceptible to infection with a virus that contains a gene encoding an EBL. Before, during, or after the administration, the animal is exposed to the virus. A determination of the efficacy of the compound is then made by, for example, detecting or measuring a change in the titer of the virus in a body fluid (e.g., blood, urine, or mucous), an organ or tissue (e.g., lung, spleen, or liver tissue), or a lavage (e.g., a lung, intestinal, bladder, or vaginal lavage).  
30           Alternatively, a reduction in one or more symptoms (e.g., fever, rash, limb paralysis) of the virus infection can be detected or measured.

The methods of the invention can be applied to a wide range of species, e.g., humans, non-human primates, horses, cattle, pigs, sheep, goats, dogs, cats, rabbits, guinea pigs, hamsters, rats, mice, and birds such as chickens, turkeys and canaries.

#### 5 In Vivo Approaches

In a preferred *in vivo* approach, the isolated compound itself is administered to the subject. Generally, the compounds will be suspended in a pharmaceutically-acceptable carrier (e.g., physiological saline) and administered orally or by intravenous (i.v.) infusion, or injected subcutaneously, intramuscularly, intrathecally, intraperitoneally, intrarectally, intravaginally, 10 intranasally, intragastrically, intratracheally, or intrapulmonarily. They can, for example, be delivered directly to a site of infection, e.g., intrapulmonarily where the infection is of the lung. The dosage required depends on the choice of the route of administration; the nature of the formulation; the nature of the patient's illness; the subject's size, weight, surface area, age, and sex; other drugs being administered; and the judgment of the attending physician. Suitable 15 dosages are in the range of 0.0001-100.0 mg/kg. Wide variations in the needed dosage are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by i.v. injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art. 20 Administrations can be single or multiple (e.g., 2-, 3-, 4-, 6-, 8-, 10-, 20-, 50-, 100-, 150-, or more fold). Encapsulation of the compound in a suitable delivery vehicle (e.g., polymeric microparticles or implantable devices) may increase the efficiency of delivery, particularly for oral delivery.

It is understood that where useful compounds are proteins, they can be delivered to cells 25 *in vivo* by administering expression vectors containing nucleic acids encoding such proteins, or cells (e.g., cells derived from the animal) transformed with such expression vectors, to the animal in which the cells reside. Such methods are well-known in the art.

#### Methods of Enhancing Immune Responses

30 The invention features methods of enhancing immune responses. The immune responses enhanced can be CD8<sup>+</sup> or CD4<sup>+</sup> T cell responses. T cell responses enhanced can be

cytokine/lymphokine-producing or cytotoxic T lymphocyte (CTL) responses.

Cytokine/lymphokine-producing responses are preferably Th1-type cytokine/lymphokine responses, e.g., interleukin (IL)-12- and IFN- $\gamma$ -producing responses. However, it may also be desirable under certain circumstances (e.g., where an antibody versus a cellular immune response is preferred) to activate responses involving the production of cytokines or lymphokines such as interleukin (IL)-2, IL-3, IL-4, IL-10, IL-13, IL-15, granulocyte-colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), or granulocyte macrophage-colony stimulating factor (GM-CSF). In so far as: (1) the methods of the invention have been shown to enhance CD4<sup>+</sup> T cell responses as well as CD8<sup>+</sup> T cell responses (Examples 6 and 7), and (2) B cell (antibody-producing) responses generally require the activity of CD4<sup>+</sup> helper T cells, the immune responses that can be enhanced by the methods of the invention include B cell responses.

These methods of the invention can be *in vivo* and are essentially the same as the above-described *in vivo* methods of inhibiting activation of cells. In addition they can be applied to the same subjects recited above for the latter method. The methods can include the additional steps of, following one or more treatments, testing for an immune response in the animal and/or characterizing a response as, for example, a T cell response, a CD4<sup>+</sup> T cell response, a CD8<sup>+</sup> T cell response, or an antibody-producing B cell response by methods familiar to those in the art.

Where it is desired to enhance immune responsiveness, supplementary agents, in addition to antibodies that substantially neutralize one or more forms of a virus of interest (see above), can be used. A method of enhancing an immune response of particular interest involves administering one or more of the above-described antibodies that bind to a viral EBL (e.g., mAbs 13E8 and 11D7) and one or more antibodies that substantially neutralize one or more forms of a virus of interest, e.g., the anti-L1R antibody.

Additional supplementary agents useful for enhancing immune response include, for example, appropriate viral antigens. An antigen can be a component of the virus with which the subject is, will be, or at is risk of being infected and that is the source of the viral EBL whose viral replication-enhancing effects the administered compound inhibits, e.g., where the method of enhancing immunity is being used for enhancing the efficiency of vaccination with live virus (e.g., vaccination against smallpox with vaccinia virus). However, additional antigens can be administered. Such antigens can be, for example, killed virus, viral proteins or peptide fragments

of viral proteins. The antigens can be administered alone or with adjuvant, e.g., cholera toxin (CT), *E. coli* heat labile toxin (LT), mutant CT (MCT) [Yamamoto et al. (1997) J. Exp. Med. 185:1203-1210] and mutant *E. coli* heat labile toxin (MLT) [Di Tommaso et al. (1996) Infect. Immunity 64:974-979]. MCT and MLT contain point mutations that substantially diminish toxicity without substantially compromising adjuvant activity relative to that of the parent molecules. Other useful adjuvants include alum, Freund's complete and incomplete adjuvant, and RIBI.

Other supplementary agents that can be used to further enhance immunity include chemokines, lymphokines, and cytokines such as MIP-1 $\alpha$ , MIP-3 $\beta$ , RANTES, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, interferon- (IFN-)  $\alpha$ ,  $\beta$ , or  $\gamma$ , granulocyte-colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), or granulocyte macrophage-colony stimulating factor (GM-CSF). In addition, antibodies specific for chemokines, lymphokines, or cytokines that inhibit an immune response of interest can be used as supplementary agents. Thus, for example, where it is desired to enhance a cellular immune response, antibodies specific for IL-4 or IL-10 may be used as supplementary agents. On the other hand, where it is desired to enhance an antibody-producing B cell response, antibodies specific for IL-12 or IFN- $\gamma$  may be employed as supplementary agents.

It is understood that all the methodologies of delivery and administration of compounds described above in the section on Methods of Inhibiting Activation of a Cell (or obvious variations of them) can be used for the delivery and administration of all the supplementary agents described herein. These supplementary agents can be administered before, after, or at the same time as the compounds. They can be administered by the same or different routes and at the same or different frequencies as the compounds.

These methods of the invention can be applied to genetic immunization. In such methods a poxvirus expression vector containing a heterologous nucleic acid sequence encoding an immunogen of interest is administered to an appropriate animal (e.g., a human, a non-human primate (e.g., a monkey or a chimpanzee), a cow, a horse, a goat, a sheep, a pig, a cat, a dog, a rabbit, a guinea pig, a hamster, a gerbil, a rat, a mouse, a chicken, or any other species recited herein) at a frequency determined to be effective by methods known in the art. In the vector, the heterologous nucleic acid sequence is operably linked to a transcriptional regulatory element (TRE). TREs include promoters and enhancers and are known in the art. As used herein, an

expression control sequence (e.g., a TRE) that is "operably linked" to a coding sequence is incorporated into a genetic construct so it effectively controls expression of the coding sequence. Poxvirus vectors of interest include vaccinia vectors, attenuated vaccinia vectors, canarypox vectors, and fowlpox vectors.

5 Routes of administration will preferably be cutaneous (e.g., by scarification), intramuscular, subcutaneous, intravenous, or intraperitoneal, or mucosal (e.g., intranasal or intrarectal). Before, simultaneous, or after the administration of the vector, one or more of the erb-B inhibitory compounds described above is administered to the animal. Any of the above-described supplementary agents can also be administered to the animal. The frequencies of  
10 administration of the compounds and supplementary agents are also readily determinable by one of skill in the art.

Since administration of an erb-B tyrosine kinase inhibitor enhanced CD4+ and CD8+ immune responses in poxvirus-infected animals (see Examples 6 and 7), the above-described genetic immunization strategy using poxvirus vectors can be effective at enhancing both cellular  
15 (CD8+ and CD4+ T cell) or B cell-mediated responses to immunogens encoded by heterologous nucleic acid sequences in the poxviral vector used for immunization. They can be used for generating therapeutic, prophylactic, or non-therapeutic/non-prophylactic immune responses to a wide variety of immunogens. These methodologies also allow the use of replication-competent poxvirus vectors (e.g., vaccinia vectors in humans), which induce more potent responses than  
20 non-replicating poxvirus vectors (e.g., canarypox vectors in humans) but which raise the concern of possible uncontrollable infection of the host animal (e.g., an immunocompromized patient), for genetic immunization. Thus, a replication-competent poxvirus vector containing an immunogen encoding sequence of interest is administered to a subject. Sufficient time is allowed for the vector to replicate sufficiently and produce sufficient immunogen to initiate a  
25 potent immune response in the subject. Methods well-known in the art can be used to establish an effective time period. One or more erb-B inhibitory compounds (with or without one or more supplementary agents) is then administered to the subject. This administration serves to both reduce the titer of the viral vector in the subject and to enhance the immune response to the immunogen.

30 Immunogens of interest can be, for example, microbial (e.g., viral, bacterial, fungal, yeast, or protozoan) antigens, parasite (e.g., nematode)-derived antigens, tumor antigens, or

proteins against which it is desired to make antibodies (polyclonal or monoclonal) for investigational, therapeutic, prophylactic, or diagnostic purposes.

Examples of relevant microbes from which immunogen-encoding nucleic acid sequences can be obtained include, without limitation, *Mycobacteria tuberculosis*, *Salmonella enteritidis*,  
 5 *Listeria monocytogenes*, *M. leprae*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Borrelia burgdorferi*, *Actinobacillus pleuropneumoniae*, *Helicobacter pylori*, *Neisseria meningitidis*, *Yersinia enterocolitica*, *Bordetella pertussis*, *Porphyromonas gingivalis*, mycoplasma, *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Chlamydia trachomatis*, *Candida albicans*, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*,  
 10 *Entamoeba histolytica*, *Toxoplasma brucei*, *Toxoplasma gondii*, *Leishmania major*, human immunodeficiency virus 1 and 2, influenza virus, measles virus, rabies virus, hepatitis virus A, B, and C, rotaviruses, papilloma virus, respiratory syncytial virus, feline immunodeficiency virus, feline leukemia virus, simian immunodeficiency virus, and all of the poxviruses recited herein. Examples of relevant microbial immunogens include, without limitation, the B subunit of heat  
 15 labile enterotoxin of *E. coli* [Konieczny et al. (2000) FEMS Immunol. Med. Microbiol. 27(4):321-332]; heat-shock proteins, e.g., the *Y. enterocolitica* heat shock protein 60 [Konieczny et al. (2000) *supra*; Mertz et al. (2000) J. Immunol. 164(3):1529-1537] and *M. tuberculosis* heat-shock proteins hsp60 and hsp70; the *Chlamydia trachomatis* outer membrane protein [Ortiz et al. (2000) Infect. Immun. 68(3):1719-1723]; the *B. burgdorferi* outer surface protein [Chen et al.  
 20 (1999) Arthritis Rheum. 42(9):1813-1823]; the *L. major* GP63 [White et al. (1999) Vaccine 17(17):2150-2161 (and published erratum in Vaccine 17(20-21):2755)]; the *N. meningitidis* meningococcal serotype 15 PorB protein [Delvig et al. (1997) Clin. Immunol. Immunopathol. 85(2):134-142]; the *P. gingivalis* 381 fimbrial protein [Ogawa, (1994) J. Med. Microbiol. 41(5):349-358]; the *E. coli* outer membrane protein F [Williams et al. (2000) Infect. Immun.  
 25 68(5):2535-2545]; influenza virus hemagglutinins and neuraminidases; retroviral (e.g., HIV) surface glycoproteins (e.g., HIV gp160/120), or retroviral tat or gag proteins.

Examples of tumors from which immunogen-encoding nucleic acids to be inserted into poxvirus vectors can be obtained include neural tissue cancer, melanoma, breast cancer, lung cancer, gastrointestinal cancer, ovarian cancer, testicular cancer, lung cancer, prostate cancer,  
 30 cervical cancer, bladder cancer, vaginal cancer, liver cancer, renal cancer, bone cancer, a hematological cell cancer, and vascular tissue cancer. Examples of relevant tumor antigens

include prostate-specific membrane antigen (PSMA) [Israeli et al. (1993) Cancer Res. 53(2):227-230], a mucin such as MUC-1, or any other antigen expressed on the surface of tumor cells.

The heterologous nucleic acid sequences in the poxvirus vectors can encode full-length immature proteins, full-length mature proteins, segments of proteins containing immunogenic epitopes, or T cell epitope-containing peptide fragments. They can encode a single polypeptide sequence or multiple (e.g., two, three, four, five, six, seven, eight, nine, ten, 11, 12, 15, 18, 20, 25, 30, or more) polypeptides. Where the heterologous nucleic acid sequence encodes more than one polypeptide, the polypeptides can be from one or more than one (e.g., two, three, four, five, six, seven, eight, nine, or ten) microbial organism(s) and/or tumor(s).

It is understood that the above-described poxvirus vector/erb-B inhibitor strategies are not limited to genetic immunization but can be applied generally to poxvirus vector gene therapy methodologies in which, instead of an immunogen, the heterologous nucleic acid sequence encodes a therapeutic molecule such as, for example, a cytokine or growth factor (such as any of those listed herein), a drug or pro-drug, or an enzyme for which a subject of interest is deficient, e.g., adenosine deaminase.

Poxviruses and their use are described in greater detail in Paoletti [(1996) Proc. Natl. Acad. Sci. USA 93:11349-11353], which is incorporated herein by reference in its entirety.

Methods to test whether a particular regimen is therapeutic for, or prophylactic against, a particular disease are known in the art. Where a therapeutic effect is being tested, a test population displaying symptoms of the disease (e.g., humans or experimental animals having smallpox) is treated with a test regimen involving of any of the above described strategies. A control population, also displaying symptoms of the disease, is treated, with a placebo or a different regimen. Disappearance or a decrease of the disease symptoms in the test subjects would indicate that the test regimen is an effective therapeutic methodology.

By applying the same strategies to subjects prior to onset of disease symptoms (e.g., experimental animals prior to deliberate infection with vaccinia virus), test regimens can be tested for efficacy as prophylactic methodologies. In this situation, prevention of, or delay in, onset of disease symptoms is tested.

The following examples are meant to illustrate, not limit, the invention.

## EXAMPLES

### Example 1. Materials and Methods

#### *Production of recombinant EGF-like domain of the SPGF D4R*

A polypeptide containing the EGF-like domain (amino acid residues 40-90 of SEQ ID NO:2) of the SPGF D4R was expressed in *E. coli* DL21 cells as inclusions and the resulting protein was compared with analogous EGF-like domains made from human EGF and mouse epiregulin (EPI). For convenience, these EGF-like domains are referred to as recSPGF, recEGF, and recEPI, respectively. The inclusions were dissolved in 6 M guanidine-HCl solution and the polypeptides were purified by Ni<sup>2+</sup>-NTA column chromatography exploiting N-terminal tag sequences in the polypeptides that contain six histidine residues. The partially purified EGF-like domains were refolded in redox buffer (2mM reduced and 1mM oxidized glutathione) and the polypeptides were further purified using RP-HPLC (reverse phase-high pressure liquid chromatography). RecSPGF (8 kDa) was compared to recEGF (6 kDa) and recEPI (5 kDa) by 15% sodium SDS-PAGE; proteins were stained with Coomassie blue (Fig. 1).

#### *Human keratinocyte and fibroblast mitogen assays*

The normal primary human dermal fibroblast line R2F [Tubo et al. (1987) Oncogene Re. 1:407-421] was cultured in "complete fibroblast medium" consisting of DMEM/F12 medium (Life Technologies, Inc., Carlsbad, CA) supplemented with 15% calf serum (HyClone, Inc., Logan, UT) and 10 ng/ml EGF. The normal primary human epidermal keratinocyte line N [Schön et al. J. Invest. Dermatol. 107:428-438] was cultured as previously described [Rheinwald et al. (2002) Mol. Cell Biol. 22:5157-5172] in "complete keratinocyte medium" consisting of GIBCO keratinocyte serum-free medium (Life Technologies, Inc.) supplemented with 30 µg/ml bovine pituitary extract (BPE), 0.2 ng/ml EGF, and 0.3 mM CaCl<sub>2</sub>.

Mitogenicity assays were performed by plating cells at low density in the relevant medium but without EGF and with a reduced amount of serum (and a reduced amount of BPE for N cells) such that their proliferation in response to a range of concentrations of added erb-B ligands could be assessed.

3000 R2F cells were plated in replicate 9 cm<sup>2</sup> culture wells in DMEM/F12 medium containing 1% calf serum. The next day, and on the 4th day after plating, the wells were fed

with medium but supplemented with recEGF or recSPGF (at various concentrations) or with complete fibroblast medium.

1000 N cells were plated in replicate 9 cm<sup>2</sup> wells in GIBCO keratinocyte serum-free medium containing BPE (15 µg/ml) and 0.3 mM CaCl<sub>2</sub>. The next day, and on the 4th day after plating, the wells were fed with this medium but supplemented with recEGF or recSPGF (at various concentrations) or with complete keratinocyte medium.

Six days after plating, the cells were detached from the culture well bottoms by trypsinization and counted. The proliferation rate was measured as population doublings per day:  $[\log_2 (\text{number of cells after 6 days} / \text{number of cells plated})] / 6$ .

For tyrosine kinase inhibition experiments, human foreskin fibroblasts (SC-J) were plated in 6-well tissue culture plates and "starved" overnight in 2% FBS (fetal bovine serum)-DMEM medium. The cells were pretreated with tyrosine kinase inhibitors for 1 hour at 37°C and then stimulated with different amounts of recSPGF. Eighteen hours later, the cells were harvested and fixed in 80% ice-cold ethanol at 4°C for 1 hour. After a single wash with phosphate-buffered saline (PBS), the cells were stained with a solution of 2.5 µg/ml propidium iodide containing 50 µg/ml RNase A at 37°C for 30 minutes. Cell cycle data were obtained by FFC analysis and analyzed using CellQuest® (Becton Dickinson, San Jose, CA) and Modfit® (Becton Dickinson) software.

#### *RecSPGF biotinylation and binding to epithelial cells analyzed by FFC*

RecSPGF was biotinylated using the ECL protein biotinylation module (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. 1 x 10<sup>5</sup> MB468 (or MB453) epithelial cells were used for each test sample. For the direct binding assay, one aliquot of cells was incubated with 1 µg anti-erb-B1 blocking antibody (mAb 528; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C for 1 hour. Different concentrations of biotinylated recSPGF in 50 µl FFC buffer (1 x PBS/2.5% FCS/0.02% NaN<sub>3</sub>) were added to the samples that were then further incubated at 4°C for 45 minutes. For competition assays, cells were incubated with 50 µl FFC buffer containing different amounts of unlabeled recSPGF, recEPI or recEGF at 4°C for 20 minutes. Biotinylated recSPGF (25 ng) was added to all samples, which were then incubated at 4°C for 30 minutes. Cells were washed in FFC buffer and bound biotinylated recSPGF was detected with streptavidin-conjugated with phycoerythrin (PE) (Molecular Probes,

Eugene, OR) by FFC. Data collected were mean fluorescence intensities (MFI). The inhibition of ligand binding is expressed as the percentage of  $(\text{MFI}_{\text{exp}} - \text{MFI}_{\text{min}}) / (\text{MFI}_{\text{max}} - \text{MFI}_{\text{min}})$  where  $\text{MFI}_{\text{exp}}$  is the MFI detected with an experimental sample,  $\text{MFI}_{\text{min}}$  is the MFI detected with a negative control sample to which no biotinylated recSPGF was added, and  $\text{MFI}_{\text{max}}$  is the MFI detected with a positive control sample to which biotinylated recSPGF was added but no inhibitor was added.

*Cell lysate preparation, immunoprecipitation, and western blotting*

HeLa cells (80%-90% confluent) in 10 cm culture dishes were stimulated with various erb-B1 ligands at a concentration of 50 ng/ml at 37°C for 10 minutes. The dishes were washed once with ice-cold PBS. The cells were then lysed directly in the dishes with 1 ml lysis buffer (25 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 0.35 trypsin inhibitor units/ml aprotinin, 5 µg/ml leupeptin, 10 mM NaF, 10 mM β-glycerophosphate and 1 mM Na<sub>3</sub>VO<sub>4</sub>) at 4°C for 30 minutes. The lysates were transferred to microfuge tubes and, after centrifugation, 0.5 ml lysate supernatant was immunoprecipitated with 1 µg of goat anti-EGFR polyclonal antibody (Santa Cruz Biotechnology, Inc.) and 10 µl gamma-bind plus™ beads (Amersham Biosciences) at 4°C overnight. The beads were washed three times and the proteins were eluted from the beads directly in 2 x SDS-PAGE loading buffer. Aliquots of whole cell lysates or immunoprecipitated samples were resolved by 7.5% SDS-PAGE and the resulting gel was blotted onto a PVDF (polyvinylidene fluoride) membrane. The membrane was blocked in Tris-buffered saline/Tween-20 (TBST) containing 2.5% bovine serum albumin (for erb-B receptors) or TBST containing 2% gelatin (for 4G10) at 37°C for at least 30 minutes. The membranes were incubated with primary antibody overnight at 4°C according to the manufacturer's instructions, washed, and incubated with 1:10,000 anti-goat IgG (for erb-B receptors) or anti-mouse IgG2b (for 4G10) HRPO (horseradish peroxidase) conjugates at room temp for 1 hour. After extensive washing, the membranes were developed using a chemiluminescence reagent kit (PerkinElmer Biosystems, Wellesley, MA) and MR film (Eastman Kodak Co., Rochester, NY).

In tyrosine kinase inhibition experiments, HeLa cells were treated with the inhibitors at 50 nM at 37°C for 30 minutes, followed by stimulation with recSPGF (50 ng/ml). Cell lysates were prepared and subjected to immunoprecipitation (as described above) with goat anti-erb-B1

polyclonal antibody (Santa Cruz Biotechnology, Inc.) and gamma-bind plus beads or anti-c-Cbl antibody-coated beads (Santa Cruz Biotechnology, Inc.) at 4°C overnight using standard methods. Beads were washed and eluted in 2 X SDS-PAGE loading buffer. Whole cell lysates or immunoprecipitates were analyzed by western blotting with either anti-EGFR or anti-c-Cbl polyclonal antibodies.

#### *Chemical crosslinking of recSPGF and erb-B receptors*

2.25 x 10<sup>6</sup> MB468 or MB453 cells were pre-treated with 0.04% NaN<sub>3</sub> at 4°C for 30 min to inhibit receptor internalization. The cells were then incubated with biotinylated recSPGF (1 µg/ml) for 30 minutes at 4°C. The culture dishes were washed with ice-cold DMEM and bis(sulfosuccinimidyl)-suberate (BS<sup>3</sup>) crosslinker was added at a concentration of 1 mM. The reaction mixtures were incubated for an additional 1 hour at 4°C with occasional shaking. After two washes with ice-cold PBS, 1 ml lysis buffer (25 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 0.35 trypsin inhibitor units/ml aprotinin and 5 µg/ml leupeptin) was added directly to the dishes followed by shaking at 4°C for 30 minutes. The lysates were transferred to microfuge tubes and, after centrifugation, the lysate supernatants were subjected to immunoprecipitation (IP) with 1 µg anti-erb-B1, anti-erb-B3, or anti-erb-B4 goat polyclonal antibodies (Santa Cruz Biotechnologies) or a 1:40 dilution of anti-erb-B2 (Cell Signaling Technology, Beverly, MA) rabbit polyclonal antibody, and 10 µl of gamma-bind plus beads. After rotating the samples at 4°C for 5 hours, the beads were washed 4 times with 1 x TBS/ 1% Triton X-100 and then boiled in 2 x SDS-PAGE loading buffer. SDS-PAGE and western blotting with streptavidin-HRPO were used to detect the biotinylated recSPGF.

#### *Affinity of recSPGF binding to EGFR*

A lactoperoxidase-catalyzed method was used for <sup>125</sup>I-labeling of recSPGF and a Chloramine T-catalyzed method was used for <sup>125</sup>I-labeling of recEGF. The specific activity of labeled recSPGF was 0.54 pm/cpm and that of recEGF was 0.34 pm/cpm. MB468 cells were plated at a concentration of 3 x 10<sup>4</sup>/0.1ml/96 well. <sup>125</sup>I- labeled recSPGF or recEGF was added at various concentrations to the cells in 50 µl of binding medium (L15 medium with 0.1% NaN<sub>3</sub>) at 4°C and incubated for 5 hours. Supernatants were harvested and the cells were quickly washed twice with 60 µl ice-cold binding medium. The supernatants and washes were then

combined. 50  $\mu$ l 0.5 N NaOH was added to each well and the plate was incubated at room temp for 1 h to lyse the adherent cells and further washed once with 60  $\mu$ l 0.5 N NaOH. The lysates and washes were combined.  $\gamma$ -counting was used to determine the amount of free ligand in the culture supernatant and the amount of bound ligand in the cell lysates. Receptor number and affinity were determined by Scatchard analysis (e.g., Current Protocols in Immunology. Eds. John E. Coligan et al., John Wiley & Sons. (2001)).

#### *Erb-B1 internalization*

HeLa cells were cultured on chamber slides and stimulated with Erb-B1 ligands (50 ng/ml) at 37°C for 10 minutes. The cells were then fixed with 3.7% formaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. The cells were incubated with 1 $\mu$ g of anti-erb-B1 mAb (Santa Cruz Biotechnology, Inc.) in 1% BSA/PBS at room temperature for 30 minutes. Bound anti-erb-B1 mAb was detected by staining with an anti-mouse Ig-FITC (fluorescein isothiocyanate) conjugate.

A431 epidermal carcinoma cells were pretreated with 50 nM inhibitors at 4°C for 30 minutes. The cells were incubated with biotinylated recSPGF (100 ng) for 30 minutes at 4°C. After washing, the cells were incubated at 37°C for 5 minutes and fixed in formaldehyde for 5 minutes at room temperature. Subsequent to streptavidin-PE staining, the MFIs of the various sample were obtained by FFC.

#### *Cells and viruses*

Vaccinia virus strain WR was propagated in L929 cells and viral titer assays were performed in Vero cells as previously described [Selin et al. (1994) J. Exp. Med. 179:1933-1943; Selin et al. (1998) J. Exp. Med. 188:1705-1715]. Unpurified vaccinia virus from tissue culture supernatants was used for all animal infections.

#### *Vaccinia virus infection*

6-8 week-old male C57BL/6 (B6) mice were injected intraperitoneally (i.p.) with 200  $\mu$ g test antibody and/or 1 mg CI-1033 in PBS. Antibody was administered only once but the CI-1033 was given each day for the 6-10 day duration of the experiment. Six hours after antibody and the first CI-1033 administration, the mice were infected intranasally with 4 x 10<sup>4</sup> PFU

(plaque-forming units) of vaccinia virus. Each day the mice were weighed and observed for symptoms of infection (1 = normal, 2 = ruffled fur; 3 = 2 plus hunched position; 4 = 3 plus little locomotion; 5 = 4 plus lethargic and minimally responsive). Viral titers were determined at the indicated time points.

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#### *RNase protection cytokine assay*

One lobe of a lung from each mouse sacrificed at various days post-infection with vaccinia virus was disrupted in RLT buffer (Qiagen, Valencia, CA) through an 18 gauge needle. Lysates were further homogenized using QIAshredder™ modules (Qiagen) and purified using an RNeasy Mini™ kit (Qiagen) according to the manufacturer's instructions. Equal amounts of total RNA (2 µg/mouse) were pooled from three mice at each time point (days 4, 6 and 8). Six µg of RNA were hybridized with probes made from the mCK-2b™ template set (BD Biosciences-Pharmingen) according to the manufacturer's instructions and protected fragments were resolved on a DNA sequencing gel. An autoradiogram was generated by exposing the gel to x-ray film.

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#### *Intracellular cytokine staining*

Single cell suspensions were prepared from spleens of infected mice treated in various ways and erythrocytes removed by lysis using a 0.84% NH<sub>4</sub>Cl solution. Vaccinia virus-specific, IFN-γ producing, CD8<sup>+</sup> T cells were detected by FFC after stimulation *in vitro* with vaccinia virus-infected fibroblasts (MC57G), uninfected control MC57G fibroblasts, or plate bound anti-CD3 (145-2C11) mAb at 5 µg/ml using the Cytofix/Cytoperm Kit Plus™ (with GolgiPlug™, BD Biosciences-Pharmingen).

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Briefly, MC57G cells were infected with the WR strain of vaccinia virus by incubation for 2 h with 5 x 10<sup>6</sup> PFU of the virus (M.O.I (multiplicity of infection) = 1.2). Subsequently, 1-2 x 10<sup>6</sup> splenocytes from the experimental mice were incubated in 96-well plates with 2.5x10<sup>5</sup> vaccinia-infected MC57G (VV-MC57G) cells or uninfected MC57G cells in the presence of 10 U/ml human recombinant IL-2 (BD Biosciences), and 0.2 µl GolgiPlug™ reagent for 5 hours at 37°C. Control cultures to detect all recently activated CD8<sup>+</sup> T cells contained anti-CD3 mAb. In these cultures splenocytes were incubated (under the same conditions described above) with

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purified anti-mouse CD3 $\epsilon$  mAb (145-2C11) coated onto the bottoms of culture wells (at a concentration of 5  $\mu$ g/ml).

Following pre-incubation with 1  $\mu$ L of Fc Block<sup>TM</sup> reagent (2.4G2 mAb) in 96-well plates containing 100  $\mu$ L of FFC buffer (HBBS (Hank's Balanced Salt Solution), 2% FCS, 0.1% NaN<sub>3</sub>), the cells were stained (20 minutes, 4°C) with combinations of fluorochrome-labeled mAbs specific for CD8 $\alpha$  (mAb 53-6.7) and CD44 (mAb IM7). Subsequent fixation and permeabilization of the cells were performed to allow intracellular access to the anti-IFN $\gamma$  mAb (XMG1.2; BD Biosciences). Freshly stained samples were analyzed using Becton Dickinson FACSCalibur and CellQuest software (San Jose, CA).

#### *Testing for the effect of CI-1033 on variola plaque and comet formation*

Confluent BSC-40 cell monolayers were either pretreated with various concentrations of CI-1033 in RPMI + 2% FBS (RPMI-2%) in triplicate for 30 minutes at room temperature or mock pretreated under identical culture conditions. Seven concentrations of CI-1033 were evaluated and three mock pretreated controls per concentration of CI-1033 were performed in 7 individual 6-well tissue culture plates. The monolayers were infected with a suspension of variola strain Solaimen such that ~50 plaques were observed in each well. Plates were incubated at 35°C in an atmosphere of 6% CO<sub>2</sub> for 1 hour and rocked at 15 minute intervals to insure an even infection of the monolayer. The inoculum was removed and the monolayer was rinsed 1x with RPMI-2%. The monolayers were over-laid with media without or with CI-1033 at the appropriate concentrations and incubated at 35°C in an atmosphere of 6% CO<sub>2</sub> for 4 days. Plaques were analyzed by immunohistochemical staining. Comets were defined as greater than two successively smaller plaques in comet-shaped association with a large plaque. The number of plaques and comets in the presence of different concentrations of CI-1033 were compared to control plates by using a Wilcoxon rank-sum test. Seven randomly selected mock-treated wells, one selected from each of seven plates, were used for comparison.

#### Example 2. The EGF-like Domain of an SPGF Stimulates Cell Growth and/or Cell Survival

Fig. 2 shows the amino acid sequence alignments of human and murine epiregulin and the orthopox viral orthologs D1L, CMP11R, C11R, and D3R from variola, camelpox, vaccinia, and monkeypox viruses, respectively. Note the high (~90% or greater) homology among viral

proteins with ~30% identity with the mammalian proteins. Within the EGF-like domain itself, the virtual identity of the viral protein sequences is evident (Fig. 2). In the 51 residue EGF-like domain (residues 40-90 of D1L (SEQ ID NO:1) and D4R (SEQ ID NO:2)), D4R differs from the vaccinia ortholog (C11R; SEQ ID NO:4) at only 3 residues. D1L from the variola major India strain (Fig. 3A; SEQ ID NO:1) differs by only two amino acids from D4R, the corresponding molecule from the variola major Bangladesh strain (Fig. 3B; SEQ ID NO:2).

A series of functional studies was conducted with recSPGF, recEPI, and recEGF. As indicated by the following experiment, recSPGF stimulates cell growth or cell survival. Human primary fibroblasts (SC-J) were starved in 2% fetal bovine serum (FBS)-containing medium for 6 hours and then stimulated with various concentrations of recSPGF, recEPI, or recEGF for 18 hours. Cell cycle analysis was performed using propidium iodide-staining FFC. From the FFC data, the percentage of cells in the S-phase of the cell cycle was calculated for each sample and plotted as a function of ligand concentration (Fig. 4). While recSPGF showed less potency than recEGF or recEPI (as assessed by the maximal percent of cells induced into S phase), recSPGF activity reached a plateau value at a lower molar concentration than the other growth factors.

Fig. 5 shows that SPGF is a long-term growth factor for both primary human keratinocytes (N cells) and human fibroblasts (R2F). The cells were cultured in standard tissue culture medium containing 10% FBS ("Complete") or in tissue culture medium containing 2% FBS and the indicated concentrations of recSPGF ("SPGF") or recEGF ("EGF") for 7 days, and total viable cell counts were performed daily. Data are expressed as the mean population doublings per day.

### Example 3. SPGF Binds to the Erb-B1 Receptor

There are four subtypes of EGFR: erb-B1, erb-B2, erb-B3, and erb-B4. To determine which erb-B receptor binds SPGF, two binding experiments were performed with biotinylated recSPGF using the MB453 (erb-B1<sup>-</sup>, -B2<sup>+</sup>, -B3<sup>+</sup>, -B4<sup>+</sup>) and MB468 (erb-B1<sup>+</sup>, -B2<sup>-</sup>, -B3<sup>+</sup>, -B4<sup>-</sup>) epithelial cell lines. The first experiment was a cross-linking analysis. MB453 or MB468 cells were incubated with biotinylated recSPGF. Crosslinking of erb-B receptors with recSPGF was performed with the crosslinking agent BS<sup>3</sup>. Lysates were then prepared and subjected to immunoprecipitation, which was followed by western blotting with HRPO-conjugated

streptavidin ("Sav-HRPO") (Fig. 6, upper panel) or with each of the erb-B receptor-specific antibodies (Fig. 6, middle panel). The expression of erb-B receptors 1-4 in these two cell lines is shown in the table presented in the bottom panel of Fig. 6. The upper panel of Fig. 6 demonstrates that recSPGF binds detectably to erb-B1 only.

Consistent with the chemical crosslinking results, Fig. 7 shows that an anti-erb-B1 mAb blocks recSPGF binding to MB468 epithelial cells. Since MB453 cells do not express erb-B1, no binding of recSPGF to them was detected (Fig. 7).

Binding inhibition experiments indicate that binding of SPGF to erb-B1 is slightly weaker than EGF but stronger than epiregulin (Fig. 8).

Affinity measurements of recSPGF binding to EGFR1-expressing cells were obtained by Scatchard analysis (Fig. 9). MB468 cells were plated into the wells of 96-well microtiter tissue culture plates. <sup>125</sup>I-labeled recSPGF was added at various concentrations, and the plates were incubated at 4°C for 5 hours. The affinity (K<sub>d</sub>) and receptor number were calculated as described in Example 1. This analysis indicated that there were approximately  $5.5 \times 10^4$  high affinity (K<sub>d</sub> = 0.14 nM) erb-B1 receptors and  $8.8 \times 10^6$  low affinity (K<sub>d</sub> = 258 nM) erb-B1 receptors per MB468 cell.

The erb-B1 receptor is normally distributed fairly uniformly over the surface of epithelial cells as evidenced by a largely homogeneous pattern of linear fluorescence obtained when unstimulated cells are stained with antibody specific for erb-B1 (Fig. 10A). However, 10 minutes after addition of recSPGF, recEPI, or recEGF, the receptor was rapidly internalized (Figs. 10B, 10C, and Fig. 10D, respectively).

Both the rapid internalization of erb-B1 activated by recSPGF and the ability of tyrosine protein kinase phosphorylation to activate IMV entry via formation of actin- and ezrin-containing cellular protrusions [Locker et al. (2000) Mol. Biol. Cell 11:2497-2511] serve to facilitate entry of a variola virion into a cell. Naturally, the initial binding of the variola virion to the cell surface prior to entry can be via, at least in part, a SPGF-erb-B1 interaction. In addition to enabling entry of virions into cells, the above-described activation events also render host cells more efficient viral replication "factories."

Example 4. The EGF-like Domain of an SPGF Stimulates Protein Tyrosine  
Phosphorylation in Cells

RecSPGF stimulated phosphorylation of EGF receptors and their cellular substrates. Whole cell lysates from HeLa cells that were either untreated (-) or treated with recSPGF ("SPGF"), recEPI ("EPI"), or recEGF ("EGF") were subjected to Western blotting (WB) with the 4G10 anti-phosphotyrosine monoclonal antibody (Fig. 11A). In addition, HeLa cell lysates were immunoprecipitated (IP) with anti-EGFR polyclonal antibodies and subjected to WB with the anti-phosphotyrosine antibody (Fig. 11B). Tyrosine residues on both the EGFR itself and various cellular substrates of it were phosphorylated in a similar, if not identical, manner by the three growth factors tested.

Example 5. Stimulation of Protein Tyrosine Phosphorylation by the EGF-like Domain of an  
SPGF is Inhibited by Quinazoline-based Compounds

A series of experiments was performed to evaluate the effect of several quinazoline-based tyrosine kinase inhibitors on SPGF-stimulated activities. Fig. 12 shows the chemical structures of three 4-anilinoquinazoline tyrosine kinase inhibitors (PD 153035, PD 168393, and CI-1033) as well the generic 4-anilinoquinazoline structure. The structure of the erb-B1 receptor kinase domain alone and in complex with one such inhibitor indicates how these 4-anilinoquinazolines bind to the ATP-binding pocket of the kinase domain [Stamos et al. (2002) J. Biol. Chem. 277:46265-46272]. As shown in Fig. 12, PD153035 and PD168393 have an identical 4-(3'-bromo-aniline) ring but differ in their R3 and R4 groups attached to the quinazoline ring. In particular, PD168393 has an acrylamide at position 6 which can alkylate erb-B1 Cys<sup>773</sup> so that the inhibitor irreversibly binds to erb-B1 at a 1:1 molar ratio. The tyrosine kinase active erb-B2 and erb-B4 molecules have a comparable cysteine residues at Cys<sup>784</sup> and Cys<sup>778</sup>, respectively, which can be targeted for modification. In contrast, PD153035 binds in a reversible manner, primarily via hydrophobic forces. CI-1033, like PD168393, has the acrylamide adduct at the 6 position (R3) and thus, like PD168393, binds irreversibly to erb-B1 via Cys<sup>773</sup>. CI-1033 has, in addition, the solubilizing morpholine side chain at the 7 position (R4). CI-1033 demonstrates IC<sub>50</sub> values of 0.8, 19 and 7 nM for erb-B1, erb-B2 and erb-B4, respectively [Allen et al. (2002) Sem. Oncol. 29:11-21].

In a first experiment, two quinazoline based compounds (PD168393 and PD153035) and a pyridopyrimidine-based compound (PD158780) were tested for their ability to inhibit tyrosine phosphorylation. All three compounds were obtained from Calbiochem, San Diego, CA, and the Calbiochem catalog shows their chemical structures. HeLa cells were plated into the wells of a 24-well tissue culture plate and the inhibitor compounds were added to a final concentration of 50 nM. The cells were cultured for 30 minutes and recSPGF (50 ng/ml) was added to all wells except a negative control sample (shown as (-) in Fig. 13); the sample shown as (+) in Fig. 13 received recSPGF but no inhibitor. The cells were then cultured at 37°C for a further 10 minutes and then, after washing, lysed. The cell lysates were resolved by 10% SDS-PAGE and the SDS-PAGE gel was blotted onto a PVDF membrane. The membrane was exposed to the 4G10 anti-phosphotyrosine mAb, binding of which was detected with a HRPO-conjugated anti-mouse IgG2b polyclonal antibody. The membranes were developed with ECL (enhanced chemiluminescence). The data in Fig. 13 show that tyrosine phosphorylation activated by recSPGF is inhibited by all three compounds (PD168393, PD153035, and PD158780).

The effect of tyrosine kinase inhibitors on recSPGF-mediated enhanced cell proliferation and/or cell survival was tested. As shown in Fig. 14A, after overnight stimulation of human fibroblasts with various concentrations of recSPGF, the number of cells entering S phase approached 8%. Pre-treatment of cells for 1 hour at 37°C with 50 nM concentrations of the indicated inhibitors blocks this increase in DNA synthesis. Fig. 14B shows that these same compounds inhibited tyrosine phosphorylation of erb-B1 (150kD band) as well as additional substrates (120kD, 80kD, 60kD and 55kD) phosphorylation of which was activated by recSPGF in HeLa cells. The Src family PTK-specific inhibitor PP2 had little ability to block the phosphorylation of these erb-B1 substrates, even when used at 10  $\mu$ M. In contrast, addition of PD168393 or CI-1033 at a concentration of 50 nM largely prevented recSPGF-triggered phosphorylation while the effect observed with PD153035 was only partial. These results suggest that reversible inhibitors of kinase activity may be less efficient at blocking protein tyrosine phosphorylation compared to irreversible inhibitors at the 50 nM concentration tested. Similar inhibition of tyrosine kinase phosphorylation was observed with phosphorylation activated by the vaccinia virus growth factor (VGF) (data not shown).

Fig. 10B shows the distribution of erb-B1 receptors in human HeLa epithelial cells prior to and after incubation with recSPGF for 15 minutes at 37°C, as detected by

immunofluorescence microscopy. In the absence of growth factor, the distribution of erb-B1 is primarily confined to the membrane. In contrast, post-SPGF exposure, erb-B1 molecules are rapidly internalized, appearing as punctate intracellular fluorescent aggregates. To assess whether tyrosine kinase inhibitors influence the ability of SPGF to down-modulate erb-B1, A431 cells were pre-treated with inhibitors, incubated with biotinylated recSPGF, and then fixed. Cell surface bound erb-B1 ligand was then visualized with streptavidin-PE and fluorescence was quantitated by FFC analysis. Fig. 14C shows that, in the absence of inhibitors, the MFI was ~110; in contrast, the irreversible erb-B inhibitors augmented the MFI ~3 fold (300-350) and the reversible erb-B1 inhibitor PD153035 increased the MFI to 240. The src family kinase inhibitor PP2 only modestly affects erb-B1 surface copy number.

The erb-B receptors are eliminated by two pathways: 1) ligand-dependent endocytosis and degradation involving a c-Cbl ubiquitin ligase mechanism [Levkowitz et al. (1999) Mol. Cell 4:1029-1040] and 2) stress-induced shuffling of chaperones associated with the erb-B receptors and involving proteasomal proteinases [Xu et al. (2001) J. Bio. Chem. 276:3702-3708]. Recent studies show that erb-B2 kinase inhibition by CI-1033 promotes down-regulation of erb-B2 via the second process [Citri et al. (2002) EMBO J. 21:2407-2417]. However, given that CI-1033 enhances erb-B1 surface expression, the findings suggest that the effects of CI-1033 on ligand-specific SPGF erb-B1 expression are not analogous to those on constitutive erb-B2 expression.

To test if 4-anilinoquinazolines influence c-Cbl interaction with erb-B receptors, inhibitor pre-treated or untreated recSPGF-stimulated HeLa cells were lysed and the lysates were immunoprecipitated with anti-erb-B1 antibodies. Subsequently, western blotting was performed with either anti-erb-B1 antisera to quantitate expression of erb-B molecules or with anti-c-Cbl antibody to assess the effect of the compounds on erb-B1 association. As shown in Fig. 14D, compared to unstimulated cells (-), the recSPGF-triggered HeLa cells (+) have less total erb-B1 due to rapid intracellular degradation. Consistent with the FFC analysis (Fig. 14C), PP2 had little influence on this process. On the other hand, PD153035, PD168393 and CI-1033 pre-treatment augmented the total erb-B1 protein immunoprecipitated despite recSPGF addition; note the several-fold increase in erb-B1 over that obtained from the recSPGF-unstimulated control cells. The latter result implies blockade of constitutive internalization or degradation of erb-B1 by the inhibitors. More importantly, recSPGF-induced c-Cbl association with erb-B was blocked by the 4-anilinoquinazolines but not by PP2. Loss of c-Cbl/erb-B1 complex formation

was not secondary to reduction in total cellular c-Cbl levels as shown by parallel c-Cbl immunoprecipitation and western blotting. Instead, these erb-B kinase inhibitors prevented the inducible association of c-Cbl with erb-B1 subsequent to SPGF binding.

5                    Example-6. *In vivo* Anti-viral Effect of a Tyrosine Kinase Inhibitor

Disruption of the VGF gene in vaccinia WR was shown to reduce pathogenicity of vaccinia virus *in vivo*, slowing viral growth by ~10,000 fold [Buller et al. (1988) J. Virol. 62:866-874]. Since, as shown above, CI-1033 blocks SPGF-stimulated erb-B1 driven cell growth or survival, receptor-mediated tyrosine phosphorylation, internalization and degradation, it seemed possible that the erb-B1 kinase inhibitor might attenuate orthopox growth factor activity *in vivo*. To test this possibility, the effect of CI-1033 on the clinical course of B6 mice given a lethal intranasal vaccinia WR challenge [Chen et al. (2001) Nat. Immunol. 2:1067-1076] was examined. As shown in Fig. 15A, all untreated animals died by day 7 after infection from a fulminant acute pneumonia. In contrast, daily i.p. administration of CI-1033 at 50 mg/kg beginning 6 h prior to infection prevented death. This treatment was as effective as a single 200 µg i.p. dose administration of anti-L1R vaccinia virus mAb (7D11) known to neutralize vaccinia intracellular mature virions (IMV) [Hooper et al. (2000) Virol. 266:329-339]. Combined administration of CI-1033 and anti-L1R was also protective. Clinical monitoring of animals post-infection showed that anti-L1R mAb or CI-1033 treatment reduced symptomology significantly but not as efficiently as the combination therapy (Fig. 15B).

A cohort of animals was sacrificed at 6 days post-infection and their lungs were examined. Differences in gross lung weight between the treatment groups are shown in Fig. 15C. Untreated animals had edematous lungs with multiple hemorrhages and weighing approximately two times those of normal, uninfected B6 mice. This pathology was somewhat attenuated by anti-L1R or CI-1033 treatment alone but dramatically ameliorated by the combination of CI-1033 plus anti-L1R. Consistent with the pathological findings, vaccinia virus titers in lungs were modestly reduced by anti-L1R or CI-1033 alone but essentially eliminated by the combination treatment when assayed on day 8. As all untreated animals died by day 8, day 7 viral lung titers were used for comparison. Fig. 15D also illustrates the results of:

30        (i) immunotherapy with the anti-SPGF mAb (13E8), which crossreacts with VGF (see co-pending U.S. Application Serial No. 10/429,685); and (ii) dual immunotherapy with anti-L1R

plus anti-SPGF mAbs. The additional two log reduction in viral titer observed with anti-L1R plus CI-1033 treatment (compared with anti-L1R mAb plus anti-SPGF mAb treatment) may be a consequence of the irreversible binding of CI-1033 [Fry et al. (1998) Proc. Natl. Acad. Sci. USA 95:12022-12027] compared with the reversible binding of the mAb. Irreversible binding of an inhibitor may result in a more complete blockade of the erb-B1 pathway than reversible binding of an inhibitor.

In combined anti-L1R mAb plus anti-SPGF mAb immunotherapy experiments (see copending U.S. Application Serial No. 10/429,685), viral titers did not decline until six days after infection. This slow time course was suggestive of the development of an adaptive T cell response to the virus rather than a virus-neutralizing effect of the antibodies. Although not shown, similar kinetics were observed with the anti-L1R mAb plus CI-1033 combination therapy. These findings suggest that CI-1033, alone or in combination with anti-L1R mAb, may augment T cell responses. To test this notion, T cell-regulated cytokine production in lungs was tested by RNase protection analysis at days 4, 6 and 8 post-infection. As shown in Fig. 16A, in mice given CI-1033 alone, the levels of interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-1 receptor antagonist (IL-1Ra) and IFN- $\gamma$  mRNA were augmented compared to untreated mice, peaking at day 6. For example, the level of IFN- $\gamma$  RNA was 4.1 fold greater on day 6 in the lungs of CI-1033-treated mice compared to those of the control-infected animals. However, no effect on the levels of interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), or macrophage migration inhibition factor (MIF) mRNA was seen in the lungs of mice given CI-1033.

Consistent with the lung cytokine level, systemic antigen-specific T cell responses to VV-MC57G cells were substantially enhanced in CI-1033 treated mice compared to control untreated mice as assessed by splenic CD8<sup>+</sup>CD44<sup>+</sup> T cell intracellular IFN- $\gamma$  production (Fig. 16B). The percentage of IFN- $\gamma$ -producing CD8<sup>+</sup>CD44<sup>+</sup> T cells activated by VV-MC57G cells increased 10-fold on day 8 post-infection. This increase corresponded to a 21-fold increase in the absolute number of IFN- $\gamma$ -producing CD8<sup>+</sup>CD44<sup>+</sup> T cells activated by VV-MC57G cells ( $1.85 \times 10^5$  vs.  $8.5 \times 10^3$  cells); this effect was due to lymphopenia in the untreated vaccinia virus-infected mice. In the same experiment, 22.7- and 48-fold increases were observed for anti-L1R and anti-L1R + CI-1033 treated mice, respectively. That anti-L1R treatment leads to more IFN- $\gamma$  cytokine RNA in the lung than the anti-L1R + CI-1033 dual therapy may be indicative of persistent viral load with the former treatment (Fig. 15D). Smaller but significant increases were

seen in the proportion of IFN- $\gamma$ -producing CD4<sup>+</sup>CD44<sup>+</sup> T cells following anti-L1R and anti-L1R + CI-1033 treatment of the mice (data not shown).

Example 7. Enhancement of *In Vivo* Immunity to Vaccinia Virus by Antibody Specific for VGF

In copending U.S. Application No. 10/429,685, it was shown that, like CI-1033, a mAb (13E8) specific for the variola SPGF D4R and cross-reactive with VGF, enhanced the protective effect of the anti-L1R mAb. Here it is shown that, as for CI-1033 (see Example 6), mAb 13E8 enhances T cell immunity to vaccinia virus *in vivo* and increases the enhancement of T cell immunity obtained with the anti-L1R mAb *in vivo*.

In that vaccinia virus is controlled *in vivo* by IFN- $\gamma$ -producing T cells, the level of which peaks between days 6 and 8 post-infection [Ruby et al. (1991) Lymphokine Cytokine Res. 10:353-358], the kinetics of virus reduction seen with the 13E8 and anti-L1R mAbs (copending U.S. Application No. 10/429,685) suggested that adaptive immunity was involved. Thus, CD8<sup>+</sup> T cell responses were examined in day 7 vaccinia virus-infected mice treated with: (a) the control 1A3 mAb, (b) the 13E8 mAb, (c) the anti-L1R mAb; or (d) a combination of the 13E8 and anti-L1R mAbs. Intracellular IFN- $\gamma$  levels were analyzed in freshly isolated spleen T cells stimulated *in vitro* for 5 h with VV-MC57G stimulator cells or with anti-CD3 mAb, which stimulates all recently activated T cells. The experiments showed increasing IFN- $\gamma$  responses in the CD44<sup>+</sup>CD8<sup>+</sup> T cells in the vaccinia-infected treatment groups compared to naive uninfected mice, in the following order: 1A3 mAb (control), 13E8 mAb, anti-L1R mAb, and anti-L1R mAb + 13E8 mAb (Fig. 17C). In this and other experiments, the spleens were enlarged in the anti-L1R mAb and the anti-L1R mAb + 13E8 mAb groups such that, for example, the total number of vaccinia virus-specific IFN- $\gamma$ -producing cells per spleen in combined anti-L1R mAb + 13E8 mAb-treated mice was 11-fold higher than the 1A3-treated mice ( $3.3 \pm 1.4 \times 10^5$  vs.  $0.3 \pm 0.1 \times 10^5$ , n= 3 per group).

Smaller but significant increases were seen in the proportion of IFN- $\gamma$ -producing CD4<sup>+</sup>CD44<sup>+</sup> T cells following anti-L1R and anti-L1R + 13E8 mAb treatment of the mice (data not shown).

The enhanced level of activated IFN- $\gamma$ -producing splenic T cells in anti-L1R + 13E8 mAb-treated mice suggested a reservoir of cells with potential to migrate to the lung to combat

pulmonary infection; note that CD44, an activation marker up-regulated upon T cell stimulation, is a recyclable receptor for hyaluronan that is involved in leukocyte homing to sites of inflammation [DeGrendele et al. (1997) Science 278:672-675]. To test this possibility, lungs were collected from uninfected or from day 7-infected and mAb-treated mice and examined by hematoxylin and eosin staining in tissue sections. Compared to lungs from uninfected mice, in which the alveolar spaces were readily discernable and bronchiolar epithelium was intact, the lungs from infected control mAb-treated mice were characterized by necrotic bronchiolar epithelia and severe alveolar edema. This was reflected in the weight of the vaccinia virus-infected lungs being about twice that of uninfected lungs ( $.35 \pm .04$  g vs.  $.21 \pm .01$  g,  $n = 3$ ). The alveolar spaces were obliterated in that they were filled with eosinophilic material; few, if any, infiltrating parenchymal cells were present. The degree of cellular infiltration increased somewhat in 13E8 mAb-treated mice, more so in anti-L1R mAb-treated mice, and most dramatically in the combined mAb-treated mice. In the double mAb-treated group, the alveolar spaces were open and bronchiolar epithelia appeared normal. The cells of the cellular infiltrate were mostly CD8<sup>+</sup> T cells. Leukocytes isolated from pooled lungs of three anti-L1R mAb-treated mice were 25% CD8<sup>+</sup> and 5% CD4<sup>+</sup> and those isolated from anti-L1R mAb plus 13E8 mAb-treated mice were 34% CD8<sup>+</sup> and 8% CD4<sup>+</sup> T cells. Thus, the non-neutralizing 13E8 mAb enhances clearance of vaccinia virus in association with augmented T cell responses in the spleen and lung.

The systemic inflammatory component of innate immunity, the so-called acute phase response, is rapidly induced when an organism's integrity is breached [Yoo et al. (2003) Proc. Natl. Acad. Sci USA 100:1157-1162]. Sentinel cells, including epithelial cells, produce IL-1 $\beta$  and/or IL-6 during this process [Schluns et al. (1997) J. Immunol. 1158:2704-2712]. Blockade of erb-B1 stimulation by the 13E8 mAb may help avoid subsequent cytokine dysregulation, and consequent immunosuppression, by virally encoded molecules [Diehl et al. (2002) Mol. Immunol. 39:531-536; Tanaka et al. (2001) J. Mol. Cell Cardiol. 33:1627-1635]. Moreover, EGF and TGF $\alpha$ , both erb-B ligands, can induce effusions *in vivo* [Ohmura et al. (1990) Cancer Res. 50:4915-4917]. ELGF, such as SPGF and VGF, may contribute analogous pathology, the inhibition of which by anti-ELGF antibodies (e.g., the 13E8 mAb) would constitute an additional therapeutic activity of such antibodies against orthopox virus infection.

Example 8. A Protein Tyrosine Kinase Inhibitor Inhibits Intracellular Formation and Intercellular Spread of Variola Virus

To examine the effect of CI-1033 on variola virus growth, a confluent monolayer of BSC-40 cells was infected with approximately 50 plaque forming units of variola strain Solaimen in the presence or absence of various concentrations of the erb-B inhibitor and cultured *in vitro* for 4 days. As shown in Fig. 18A, increasing concentrations of CI-1033 dramatically reduce the size of the individual plaques and comet formation but had a minimal effect on plaque number. The effect on plaque size and comet formation was titrable (Figs. 18A, 18B and 18C); at the highest concentration of CI-1033 (10  $\mu$ M), extremely small plaques were only faintly visible. The total number of plaques at each concentration did not significantly differ when compared to seven randomly selected control wells (Figs. 18A and 18B).

Comets are indicative of extracellular enveloped virus (EEV) formation and dissemination [Payne (1980) J. Gen. Virol. 50:89-100]. Fig. 18C shows a statistically significant decrease in the number of comets ( $p < 0.05$ ) at CI-1033 concentrations of 500 nM or greater. These aggregate results suggest that CI-1033 does not block primary infection but rather viral morphogenesis of EEV. Specifically, the release of EEV appears sensitive to inhibition.

A slightly less profound effect was seen on plaque size, qualitatively visible at the higher concentrations of CI-1033, indicating an effect on cell-associated enveloped virus (CEV) formation. Orthopox viruses use both microtubule and actin filaments for egress [Hollinshead et al. (2001) J. Cell Biol. 154:389-402; Smith et al. (2002) J. Gen. Virol. 83:2915-2931] with CEV inducing actin tails to eject themselves from the cell. As erb-B1 activation reorganizes the actin microfilament system [Lynch et al. (2003) J. Biol. Chem. 278:21805-21813], it appears likely that both cell-associated virus and extracellular enveloped virus release is blocked, retarding secondary virus cell-to-cell spread and hence limiting the size of individual plaques.

The more profound related effect on long range dissemination of virus via EEV (as shown by inhibition of comet formation) potentially suggests an additional mechanism of interference. Because erb-B endocytosis is also linked to the actin cytoskeleton [Lynch et al.], erb-B1 kinase inhibition may serve to maintain surface EGFR expression via this mechanism as well. The ability of CI-1033 to block erb-B activation by cellular erb-B ligands or trans-stimulation of erb-B1 via other receptors, as well as its downstream effects on cytoskeletal

elements used for poxviruses egress, likely accounts for the profound effect on plaque morphogenesis not observed with anti-SPGF mAb (data not shown).

In sum, combination immunotherapy/chemotherapy is highly efficient at reducing viral titer and stimulating T cell immunity. While the invention is not limited by any particular mechanism of action, it is possible to speculate that the latter may be a consequence of preventing the elaboration of anti-inflammatory viral products as well as later rounds of viral DNA replication in epithelial cells dependent on the viral growth factor [Alcami (2003) *Nat. Rev. Immunol.* 3:36-50; Seet et al. (1998) *Ann. Rev. Immunol.* 21:377-423]. The use of host cellular signaling pathway blockade as a target for anti-viral chemotherapy is distinct from other approaches typically directed against pathogens themselves. One advantage of the host-targeted strategy is that drug resistance cannot develop. Furthermore, unlike with anti-SPGF mAbs, where crossreactivity with other closely-related pathogenic factors (i.e., VGF vs. SPGF) is not guaranteed, the erb-B tyrosine kinase inhibitors are potentially able to block immunologically distinct ligands to various erb-B protein tyrosine family numbers. This broader target activity is relevant since, for example, VGF and myxoma virus growth factor (MGF) bind distinct erb-B receptors [Tzahar et al. (1998) *EMBO J.* 17:5948-5963].

It appears likely that administration of CI-1033 alone would be sufficient to retard "real life" infection by orthopox (e.g., natural or bioterrorist spread of smallpox) in which the dose of orthopox particle inhalation is likely to be significantly less than that employed in the present studies. That CI-1033 augments T cell responses while reducing infectious symptomatology also suggests its use: (a) as an immune stimulant in combination with vaccinia vaccination; and/or (b) in treatment of complications of vaccination.

In addition to poxviruses and their erb-B-directed growth factors, hepatitis B virus and Epstein-Barr virus have genes that encode products that dysregulate erb-B1 transcription [Menzo et al. (1993) *Virology* 196:878-882; Miller et al. (1995) *J. Virol.* 69:4390-4398]. Moreover, RNA tumor viruses such as avian erythroblastosis virus exploit erb-B1 signaling [Adelsman et al. (1996) *J. Virol.* 70:2533-2544]. Furthermore, functional EGF receptors have been reported to be necessary for efficient reovirus and human cytomegalovirus (CMV) infection of host cells [Strong et al. (1993) *Virology* 197:405-411; Wang et al. (2003) *Nature* 424:456-461]. These findings collectively suggest that multiple viral infections may be coupled to the erb-B-mediated

signaling network. Epithelial cells, which constitutively or inducibly express erb-B receptors, are components of the innate sentinel cell system in the body [Yoo et al. (2003) Proc. Natl. Acad. Sci USA 100:1157-1162]. It is possible that erb-B inhibitors will have a wider application in infectious diseases than in orthopox virus infection. Development of chemical inhibitors of cellular signaling pathways exploited by viral pathogenic factors may offer a new approach toward infectious disease control in general.

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A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

**WHAT IS CLAIMED IS:**

1. A method comprising:
  - (a) identifying an animal as likely to have been, or as likely to be, exposed to a virus, wherein the virus comprises a gene encoding a viral erb-B ligand; and
  - (b) treating the animal with a compound that inhibits the activity of an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase.
2. The method of claim 1, wherein the animal is identified as having been infected, before, during or after step (b), with the virus.
3. The method of claim 1, wherein the viral erb-B ligand is a poxvirus erb-B ligand.
4. The method of claim 3, wherein the poxvirus is an orthopox virus.
5. The method of claim 4, wherein orthopox virus is variola major.
6. The method of claim 4, wherein the orthopox virus is variola minor.
7. The method of claim 4, wherein the orthopox virus is monkeypox virus.
8. The method of claim 4, wherein the orthopox virus is vaccinia.
9. The method of claim 1, wherein the erb-B ligand is an epiregulin-like growth factor (ELGF).
10. The method of claim 9, wherein the ELGF is smallpox growth factor (SPGF).
11. The method of claim 9, wherein the ELGF is vaccinia growth factor (VGF).

12. The method of claim 1, wherein the erb-B tyrosine kinase is erb-B1 tyrosine kinase.

13. The method of claim 1, wherein the compound is a non-agonist antibody that binds to the erb-B tyrosine kinase.

14. The method of claim 1, wherein the compound is a non-agonist erb-B ligand or a non-agonist fragment of an erb-B ligand.

15. The method of claim 1, wherein the compound is a small molecule erb-B tyrosine kinase inhibitor.

16. The method of claim 15, wherein the small molecule erb-B tyrosine kinase inhibitor is a quinazoline-based compound.

17. The method of claim 15, wherein the small molecule erb-B tyrosine kinase inhibitor is a pyridopyrimidine-based compound.

18. The method of claim 15, wherein the small molecule erb-B tyrosine kinase inhibitor is a quinoline-3-carbonitrile-based compound.

19. The method of claim 15, wherein the small molecule erb-B tyrosine kinase inhibitor is pyrrolopyrimidine-based compound.

20. The method of claim 16, wherein the quinazoline-based compound is a 4-anilinoquinazoline.

21. The method of claim 15, wherein the small molecule erb-B tyrosine kinase inhibitor is an irreversible inhibitor of the erb-B tyrosine kinase activity.

22. The method of claim 21, wherein the irreversible inhibitor is CI-1033.

23. The method of claim 16, wherein the quinazoline-based compound is PD168393, PD160678, PD160879, or PD174265.

5 24. The method of claim 16, wherein the quinazoline-based compound is PD153035, ZD1839, GW572016, GW974, OSI-774, or AG1478,

25. The method of claim 17, wherein the pyridopyrimidine-based compound is PD69896, PD153717, or PD158780.

10 26. The method of claim 18, wherein the quinoline-3-carbonitrile-based compound is EKB-569.

27. The method of claim 19, wherein the pyrrolopyrimidine-based compound is  
15 CGP59326A.

28. The method of claim 1, wherein the animal is a human.

29. The method of claim 1, further comprising administering to the animal an  
20 antibody that has the ability to substantially neutralize one or more forms of the virus.

30. The method of claim 29, wherein the antibody binds to the intracellular mature virion (IMV) form, the extracellular enveloped virus (EEV) form, or the cell-associated envelope virus form (CEV) of an orthopox virus.

25 31. The method of claim 30, wherein the orthopox virus is variola major.

32. The method of claim 30, wherein the orthopox virus is variola minor.

30 33. The method of claim 30, wherein the orthopox virus is vaccinia.

34. The method of claim 30, wherein the orthopox virus is monkeypox virus.

35. The method of claim 1, wherein the virus is a poxvirus expression vector and further comprises: (a) a heterologous nucleic acid sequence encoding an immunogen; and (b) a transcriptional regulatory element (TRE), wherein the TRE is operably linked to the heterologous nucleic acid sequence.

36. The method of claim 35, wherein the poxvirus expression vector is a vaccinia virus expression vector or an attenuated vaccinia virus expression vector.

37. The method of claim 35, wherein the poxvirus expression vector is a canarypox virus or a fowlpox virus vector.

38. The method of claim 2, wherein the treatment enhances an immune response to the virus in the animal.

39. The method of claim 38, wherein the immune response is a T cell response.

40. The method of claim 39, wherein the T cell response is a CD8+ T cell response.

41. The method of claim 39, wherein the T cell response is a CD4+ T cell response.

42. The method of claim 39, wherein the T cell response is an interferon- $\gamma$ -producing T cell response.

43. The method of claim 38, wherein the immune response is an antibody-producing B cell response.

44. A method comprising:

(a) identifying an animal susceptible to infection by a virus that comprises a gene encoding an erb-B ligand; and

(b) treating the animal with (i) an antibody that substantially neutralizes one or more forms of the virus or a vaccine that stimulates an immune response against the virus and (ii) a compound that inhibits an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase.

45. An in vitro method comprising:

(a) providing an isolated compound that binds to an erb-B tyrosine kinase and inhibits the activity of tyrosine kinase activity or activation of the tyrosine kinase of the receptor;

(b) contacting the compound with a cell that expresses the erb-B tyrosine kinase; and

(c) before, simultaneous with, or after step (b), contacting the cell with a viral erb-B ligand or a functional fragment of the ligand.

46. The method of claim 45, wherein the compound reduces the activation of the erb-B tyrosine kinase on the cell by the ligand or fragment.

47. The method of claim 44, further comprising determining whether the compound reduces activation of the erb-B tyrosine kinase on the cell by the ligand or fragment.

48. A method of determining whether a compound is an antiviral compound, the method comprising:

(a) providing a compound that inhibits an erb-B tyrosine kinase activity;

(b) administering the compound to an animal susceptible to infection with a virus that comprises a gene encoding an erb-B ligand.

(c) before, during, or after step (b), exposing the animal to the virus; and

(d) determining whether the compound reduces a symptom of viral infection in the animal.

49. A method comprising:

(a) identifying an animal susceptible to infection by a virus that comprises a gene encoding an erb-B ligand; and

(b) treating the animal with an (i) antibody that binds to the ligand and (ii) an antibody that substantially neutralizes one or more forms of the virus or a vaccine that stimulates an immune response against the virus.

50. The method of claim 49, wherein the animal is identified as having been infected, before, during or after step (b), with the virus.

51. The method of claim 50, wherein the treatment enhances an immune response to the virus.

52. The method of claim 51, wherein the immune response is a T cell response.

53. The method of claim 52, wherein the T cell response is a CD8+ T cell response.

54. The method of claim 52, wherein the T cell response is a CD4+ T cell response.

55. The method of claim 52, wherein the T cell response is an interferon- $\gamma$ -producing T cell immune response.

56. The method of claim 51, wherein the immune response is an antibody-producing B cell response.

57. The method of claim 50, wherein the virus is a poxvirus expression vector and further comprises: (a) a heterologous nucleic acid sequence that encodes an immunogen; and (b) a transcriptional regulatory element (TRE), wherein the TRE is operably linked to the heterologous nucleic acid sequence.

5

58. The method of claim 57, wherein the poxvirus expression vector is a vaccinia virus expression vector or an attenuated vaccinia virus expression vector.

10

59. The method of claim 56, wherein the poxvirus expression vector is a canarypox virus or a fowlpox virus vector.

60. The method of claim 49, wherein the antibody that binds to the ligand is the 13E8 monoclonal antibody (ATCC accession no. PTA-5040).

15

61. The method of claim 49, wherein the antibody that binds to the ligand is the 11D7 monoclonal antibody (ATCC accession no. PTA-5039).

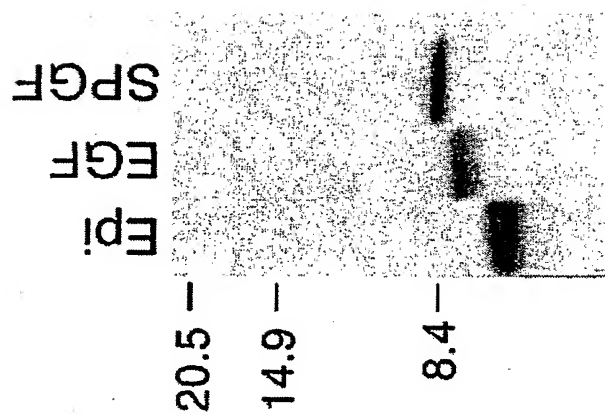


Fig. 1

D1L [Variola major]  
CMP11R [Camelpox]  
C11R [Vaccinia]  
D3R [Monkeypox]  
epiregulin [human]  
epiregulin [mouse]

-----MSMKYLMLEFAAMIIRSA-----NSGNAIETTLSEITNTT  
-----MSMKYLMLEFATMIIRSA-----DSGNAIETTSPEITNTT  
-----MSMKYLMLEFAAMIIRSA-----DSGNAIETTLPEITNATT  
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LEADER SEQUENCE

D1L [Variola major]  
CMP11R [Camelpox]  
C11R [Vaccinia]  
D3R [Monkeypox]  
epiregulin [human]  
epiregulin [mouse]

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DIPATRLCGPEGDGYCLHGDCCTHARDINGMYCRC SHGYTGIRCOHVVLVDYQORSEKPNNTT  
DIPATRLCGPEGDGYCLHGDCCTHARDIDGMYCRC SHGYTGIRCOHVVLVDYQORSEKPNNTT  
DIPATRLCGPEGDGYCLHGDCCTHARDIDGMYCRC SHGYTGIRCOHVVLVDYQORSEKPNNTT  
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AQVQITKCSSDMDGYCLHGQCCTYLVDMREKFCRCCEVGYTGIRCOHVVLVDYQORSEKPNNTT

EGF

D1L [Variola major]  
CMP11R [Camelpox]  
C11R [Vaccinia]  
D3R [Monkeypox]  
epiregulin [human]  
epiregulin [mouse]

TSYIPSPGIVLVHVS--IIVCCLLFVYRFTTRTNKLPLODMVVP-----  
TSYIPSPGIVLVHVG---IIMCCLLSVYRFTTRTNKLPLODMVVP-----  
TSYIPSPGIMLVHVGIIITCCLLSVYRFTTRTNKLPLODMVVP-----  
TSYIPSLGIVLVHVG---IITCCLLSVYRFTTRT-KLPIQDMVVLVFL-----  
--ALTVILLIILFLITVVG---STYYFCRWYRNKSKPEKKEYERTVSGDPELPQV  
--ALTVILLIILFLIITAG---CIYYFCRWYKNRKSREERYERTVSGDPVLPQV

TRANSMEMBRANE

Fig. 2

Fig. 3A

MSMKYLMLLFAAMIIRSFANSGNAIETTLSEITNTTTDIPAIRLCGPEG**DDRY**CFHGI  
CIHARDIDGMYCRCSHGYTGIRCQHVVLVDYQRSEKPNTTTSYIPSPGIVLVLLVS  
IIVCCLLFVYRFTRRTNKLPLQDMVVP

Fig. 3B

MSMKYLMLLFAAMIIRSFANSGNAIETTLSEITNTTTDIPAIRLCGPEG**NGY**CFHGI  
CIHARDIDGMYCRCSHGYTGIRCQHVVLVDYQRSEKPNTTTSYIPSPGIVLVLLVS  
IIMCCLLFVYRFTRRTNKLPLQDMVVP

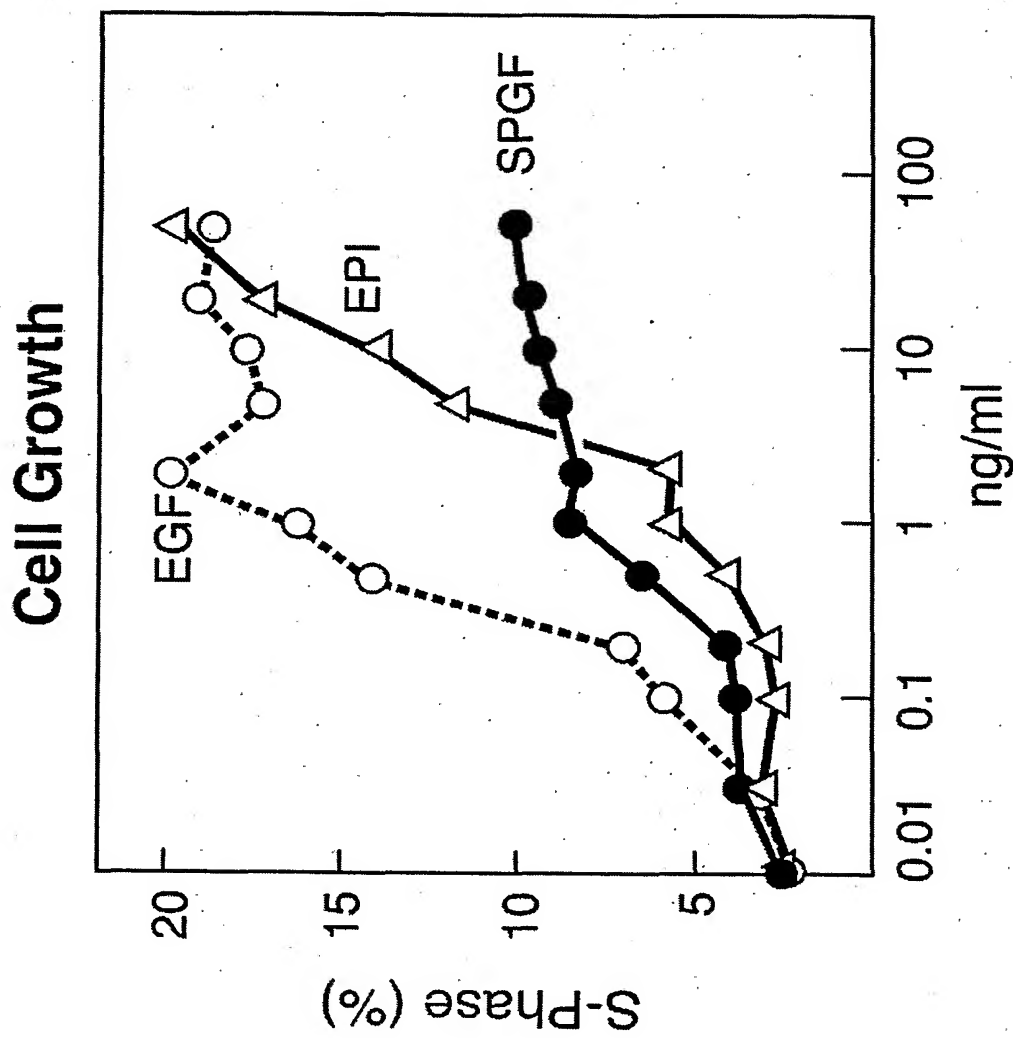


Fig. 4

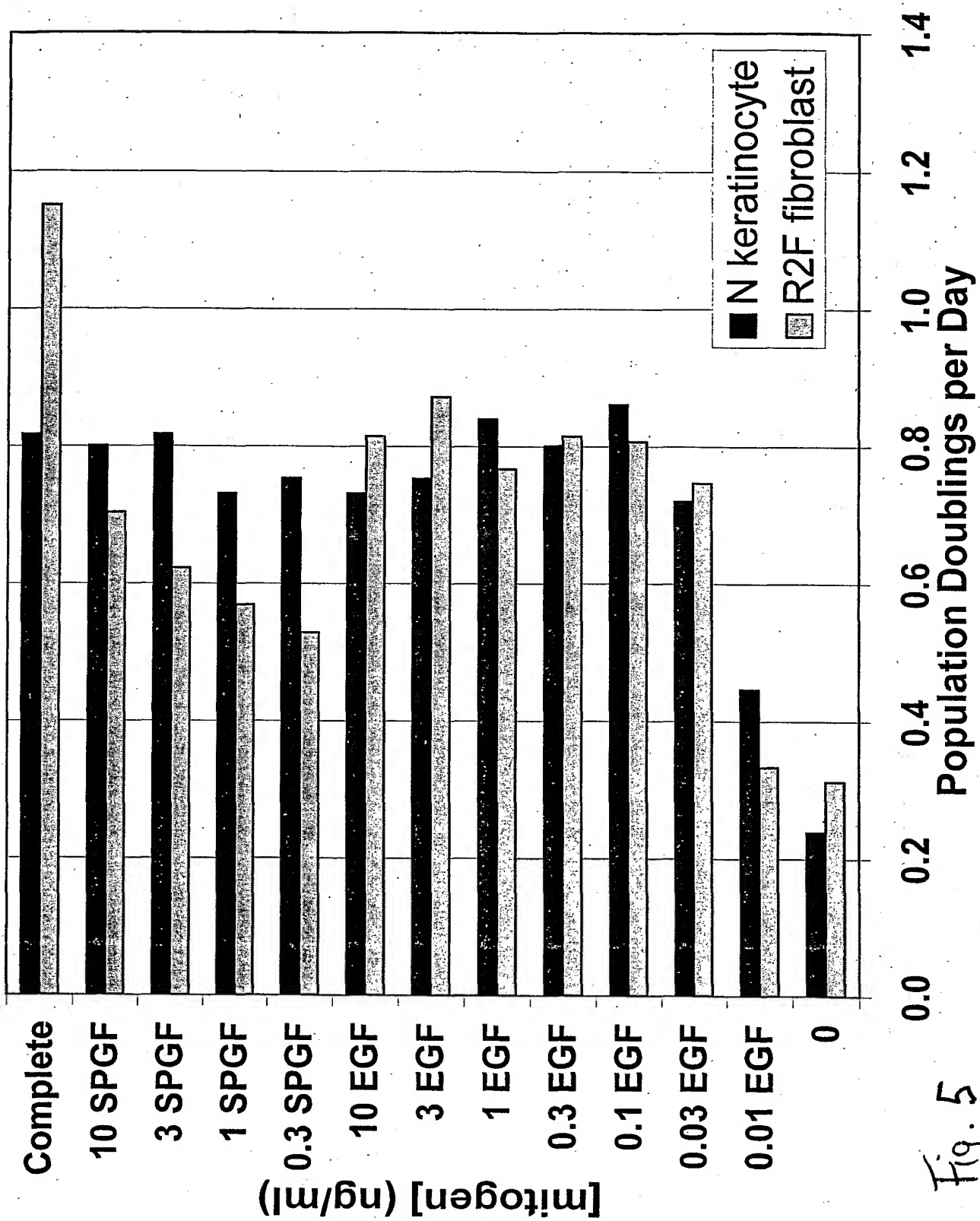


Fig. 5

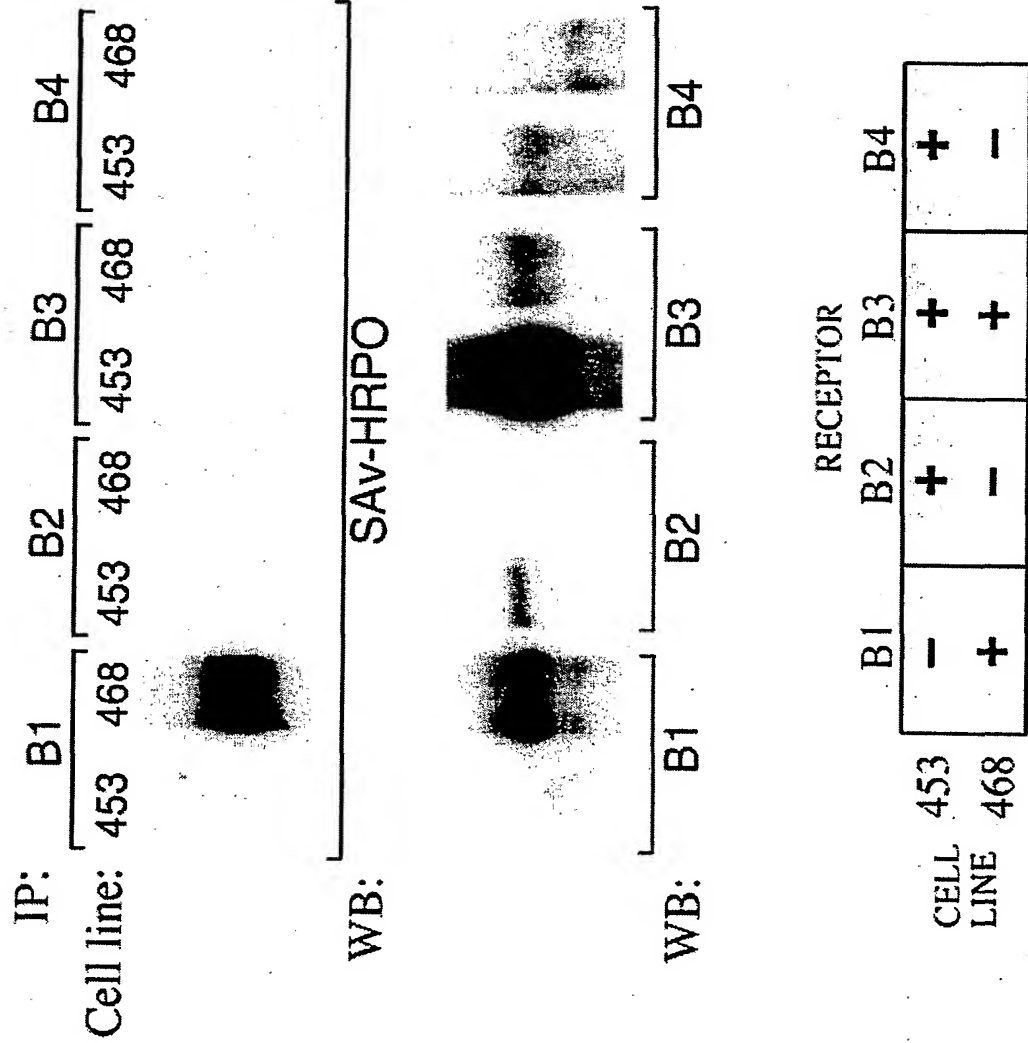


Fig. 6

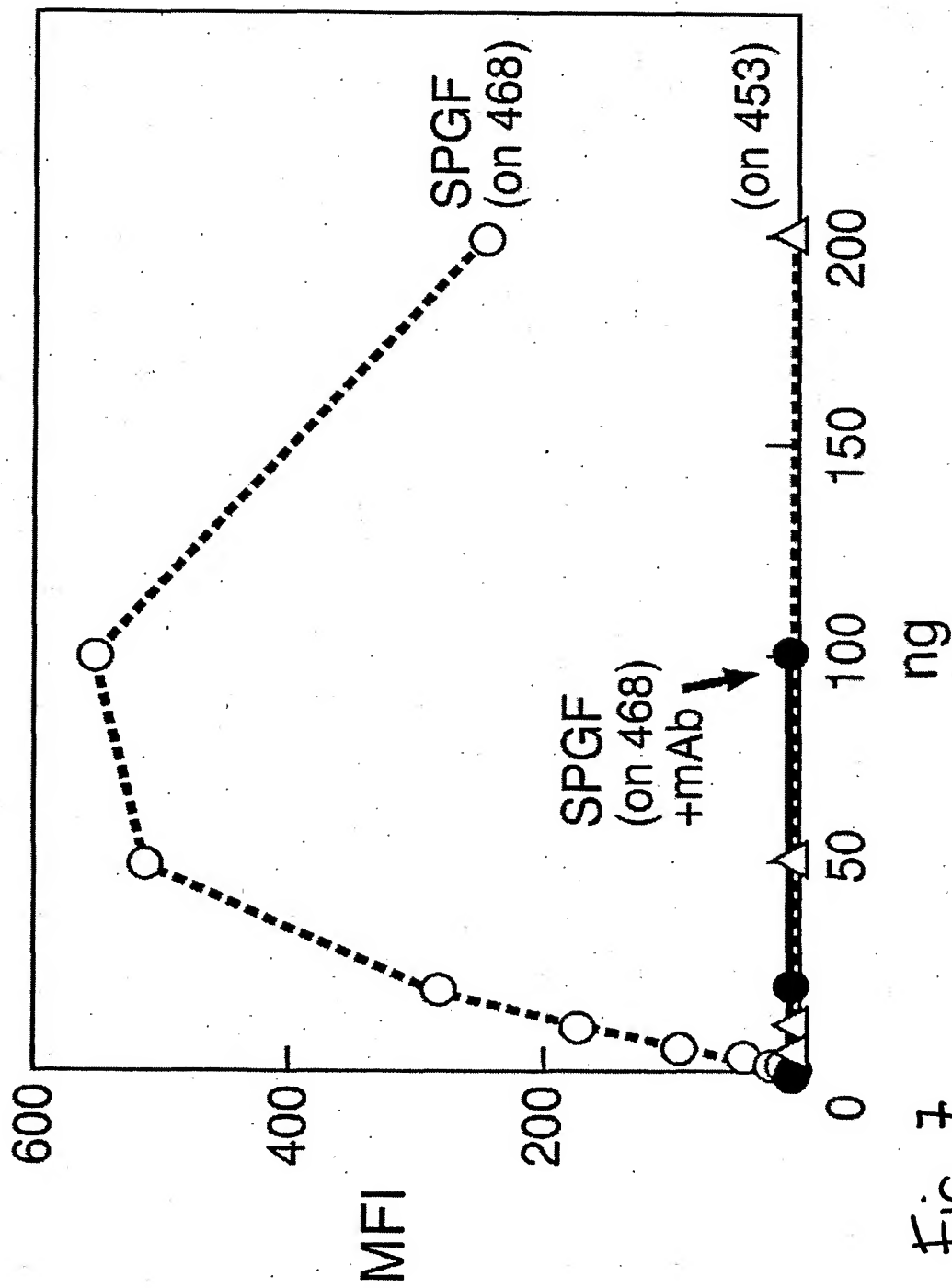


Fig. 7

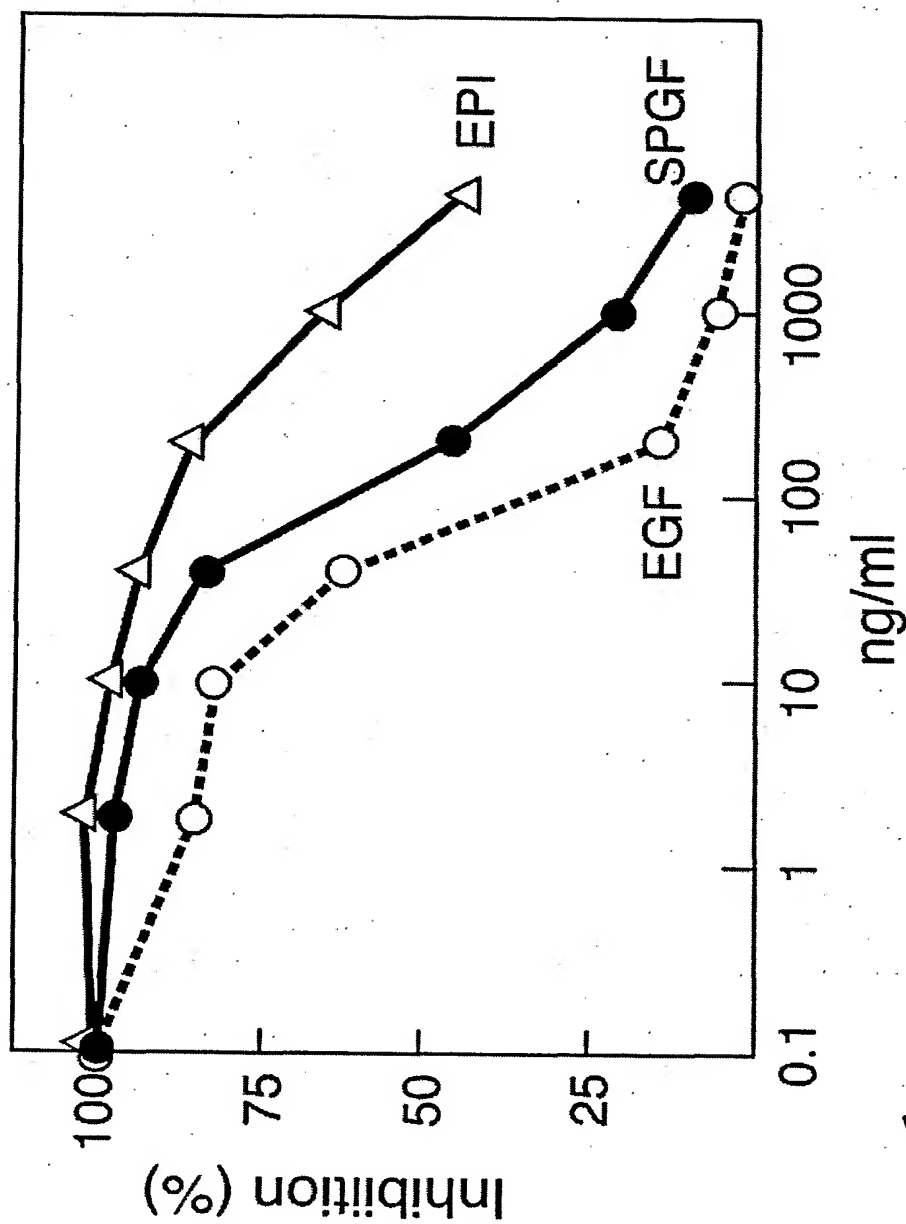
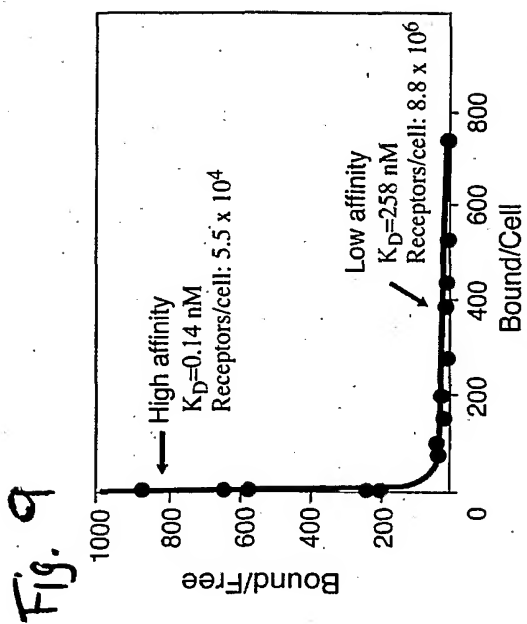


Fig. 8



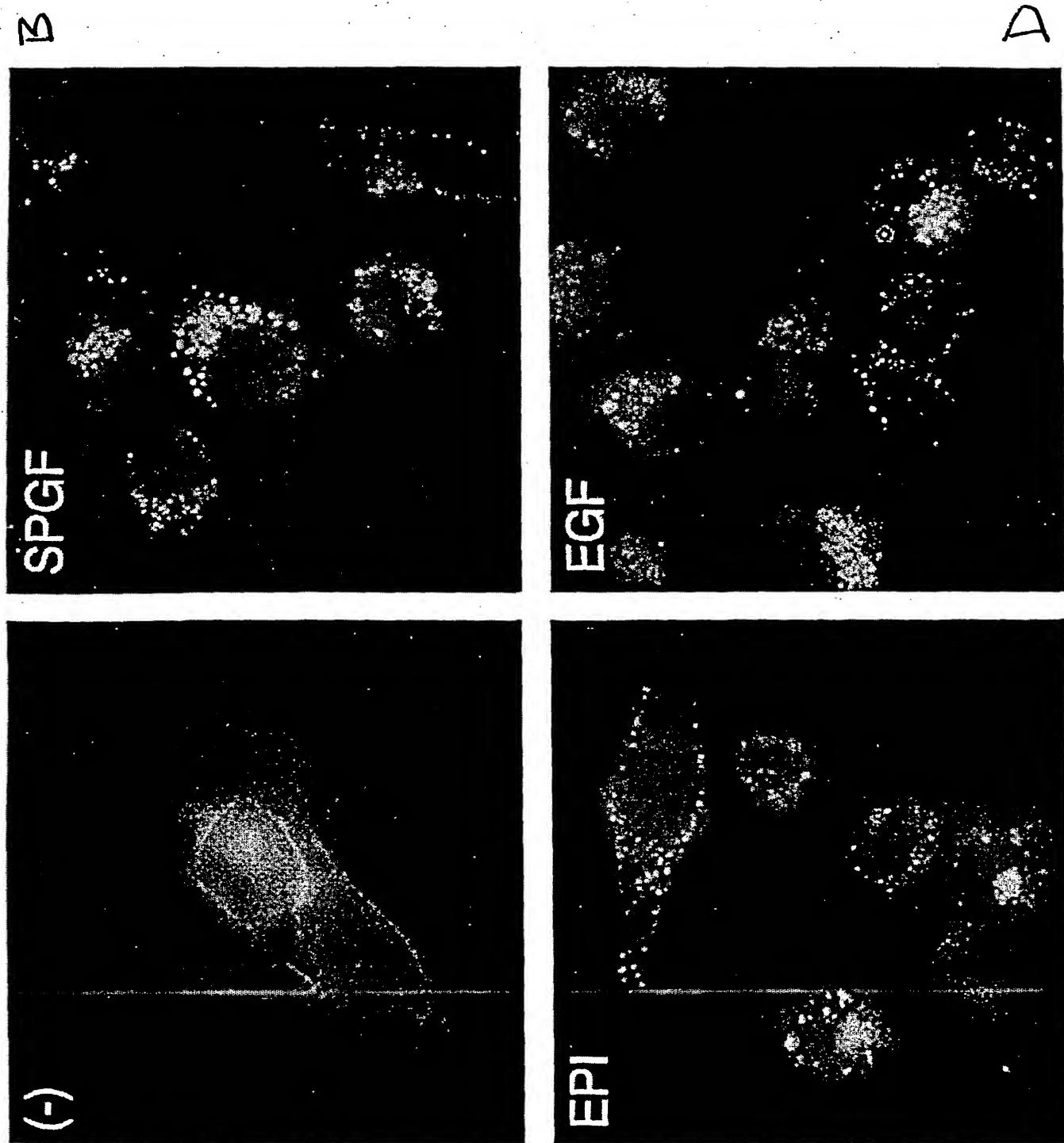


Fig. 10

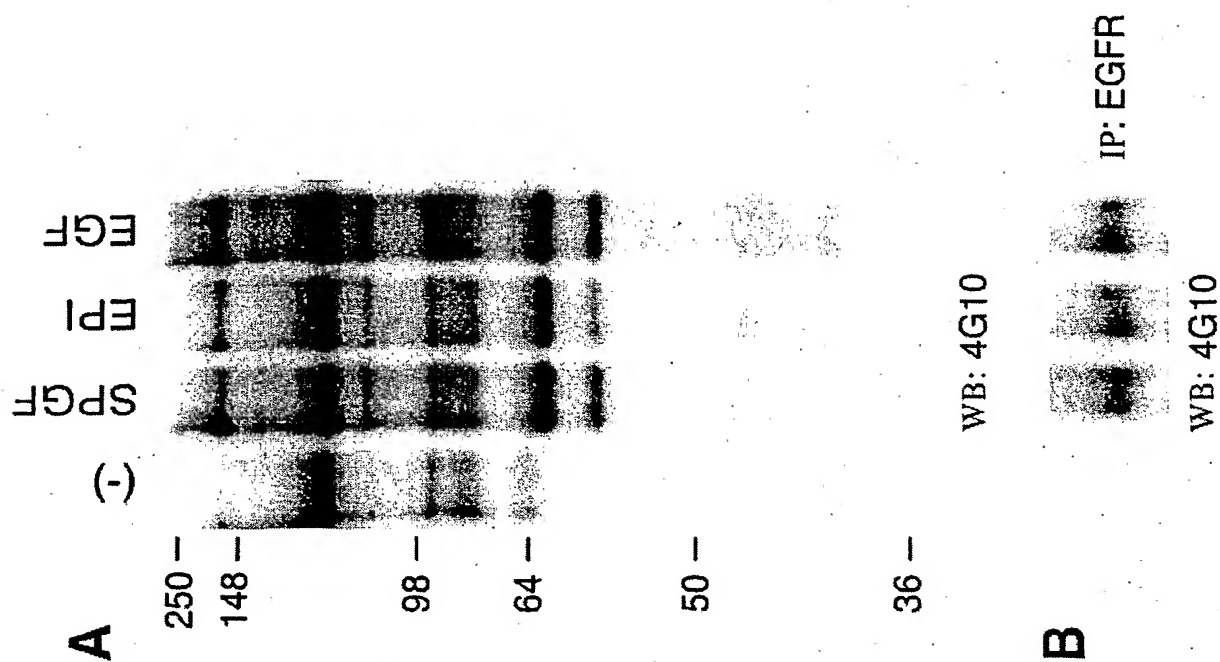
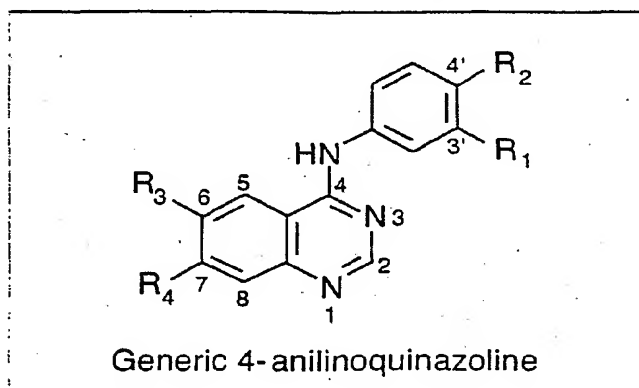
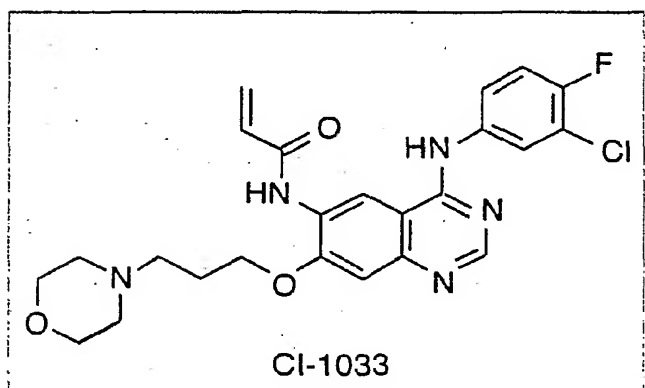
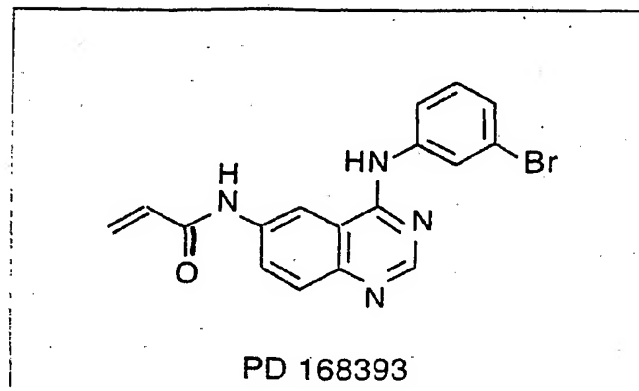
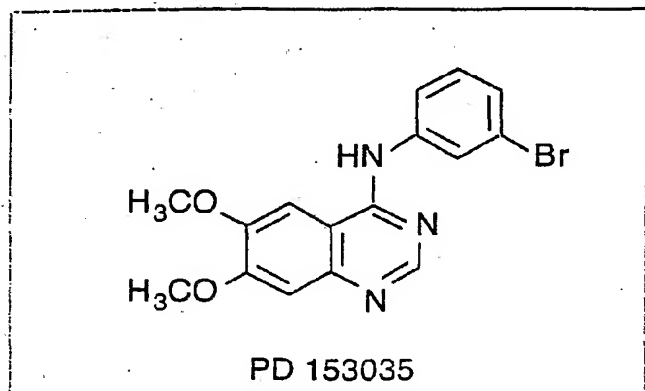


Fig. 11

Fig. 12



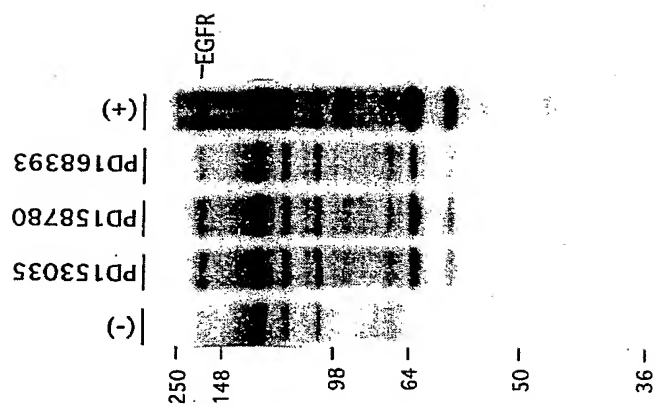


Fig. 13

Fig. 14

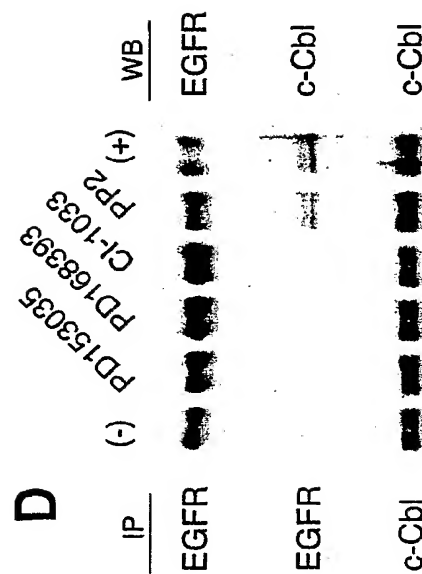
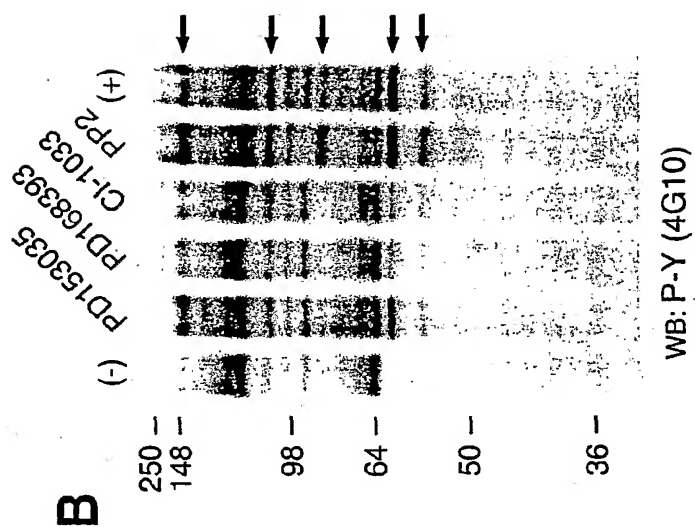
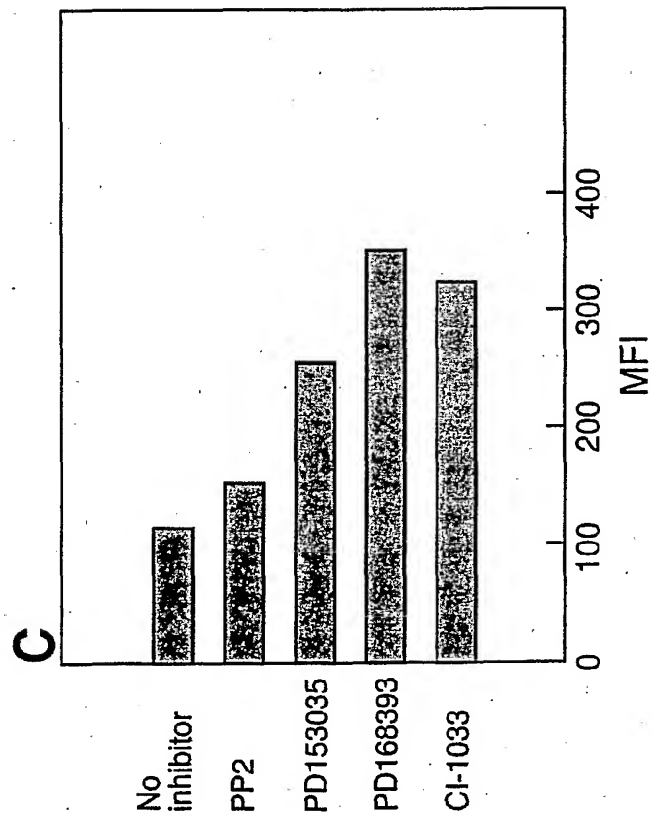
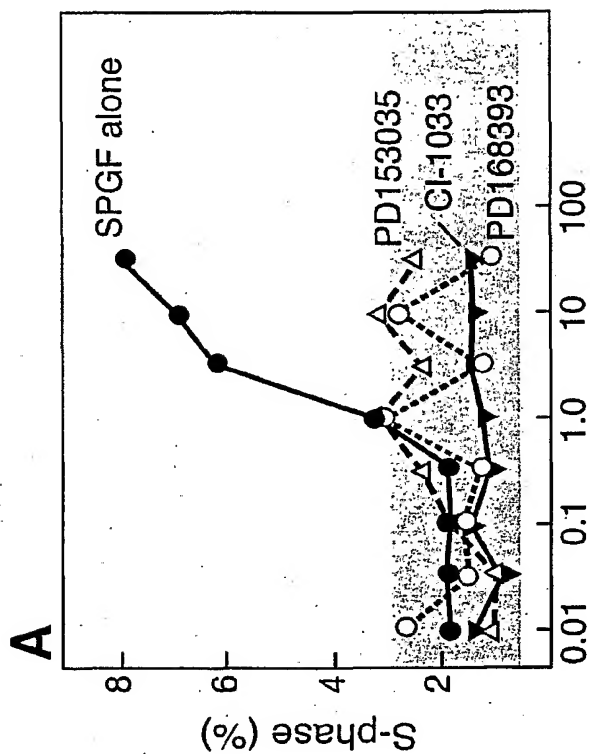


Fig. 15

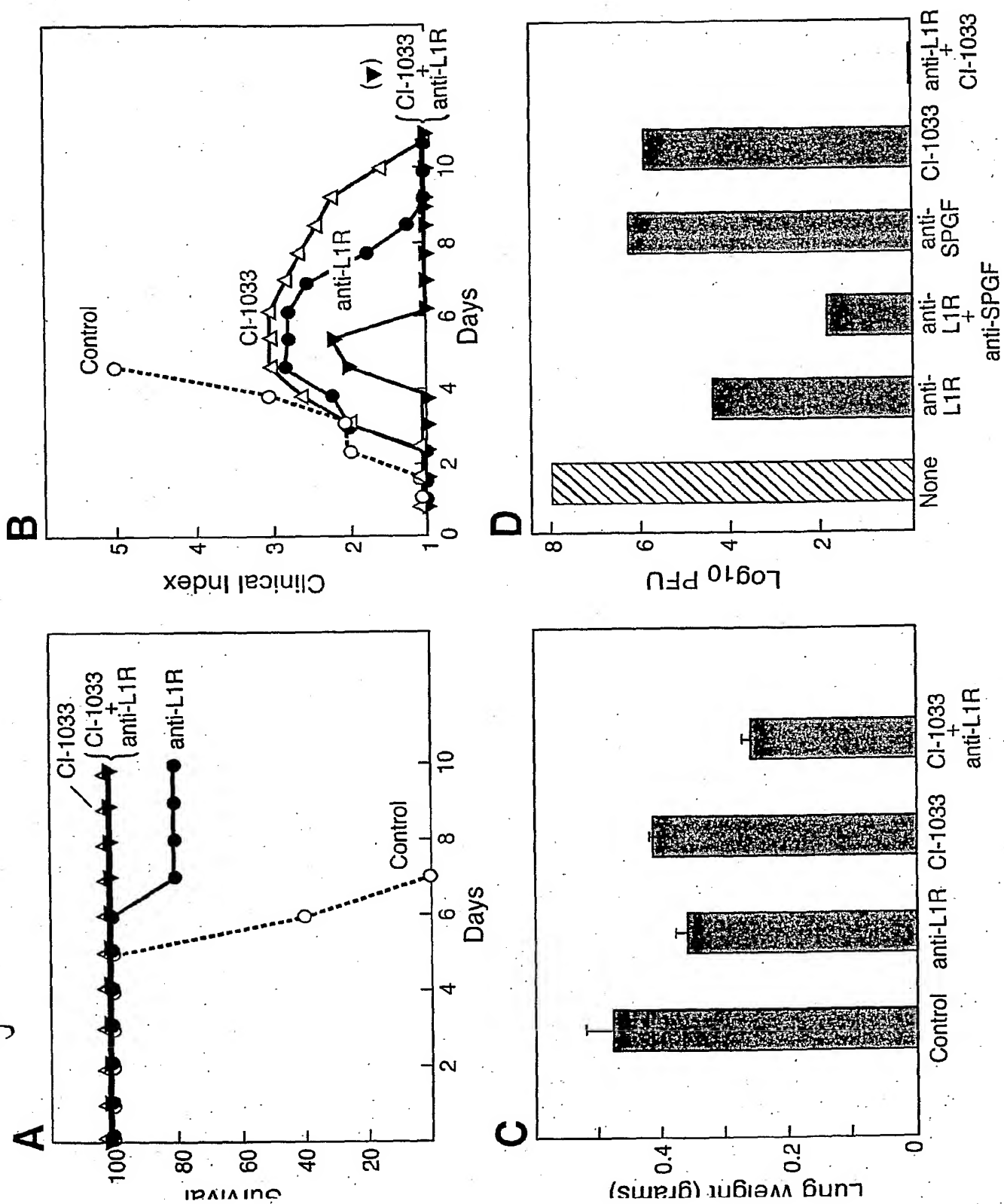


Fig. 16  
A

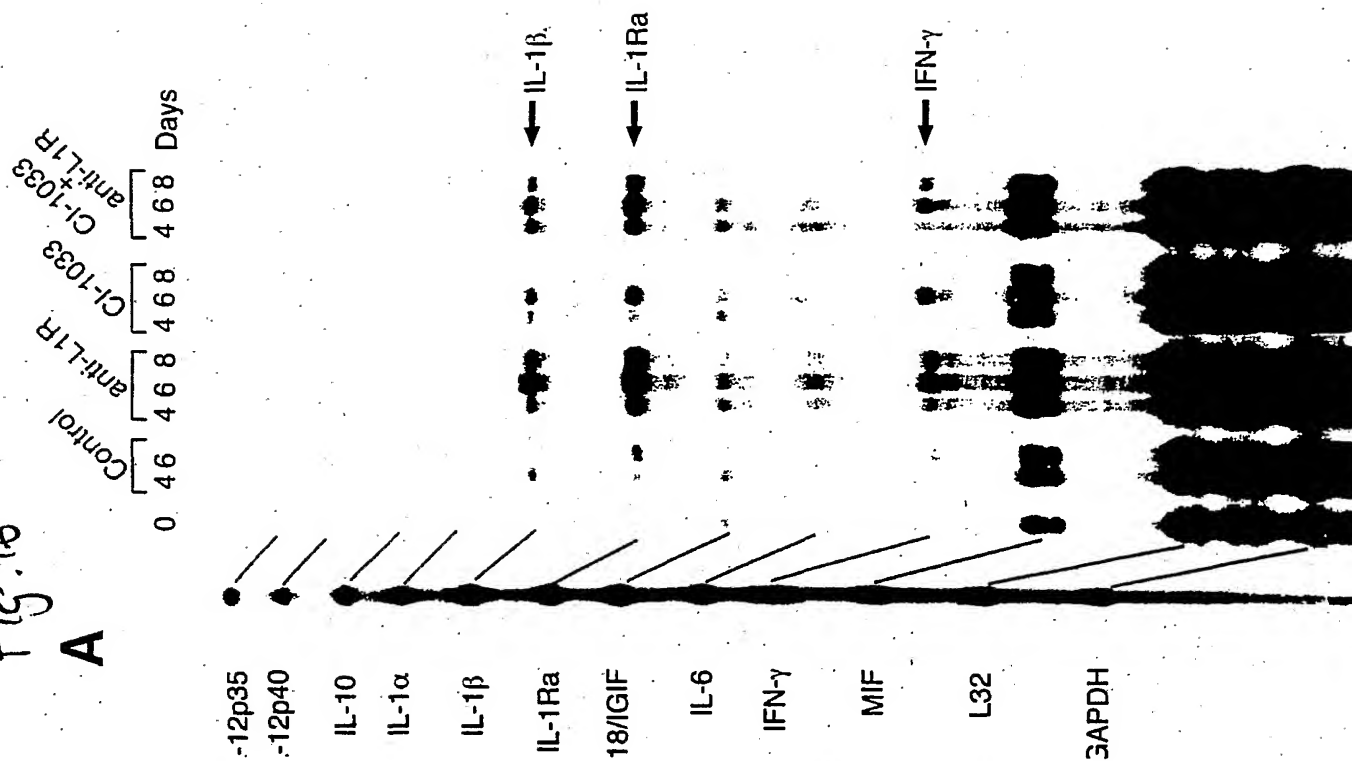
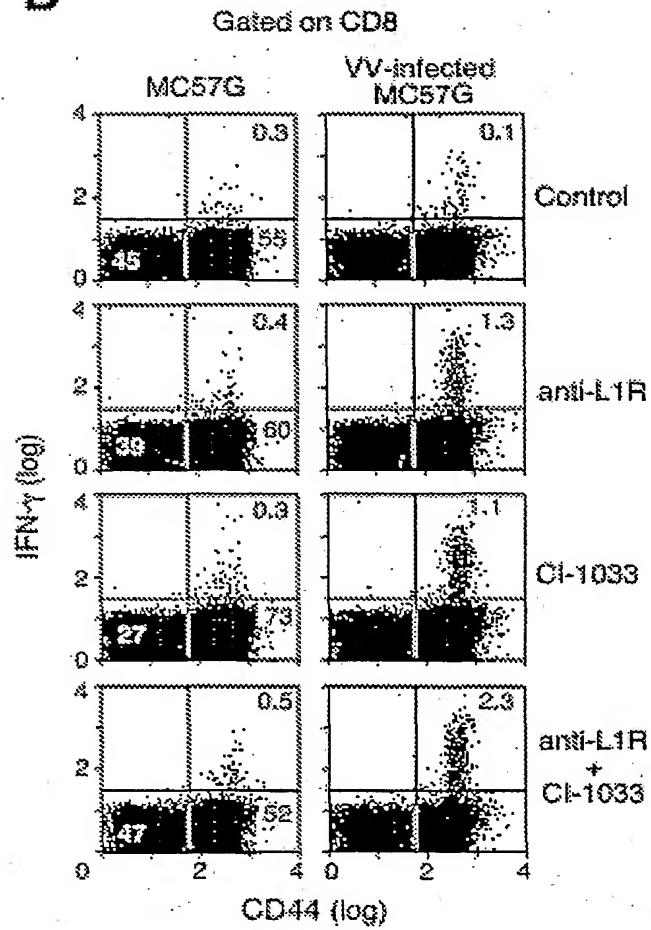


Fig. 16

**B**

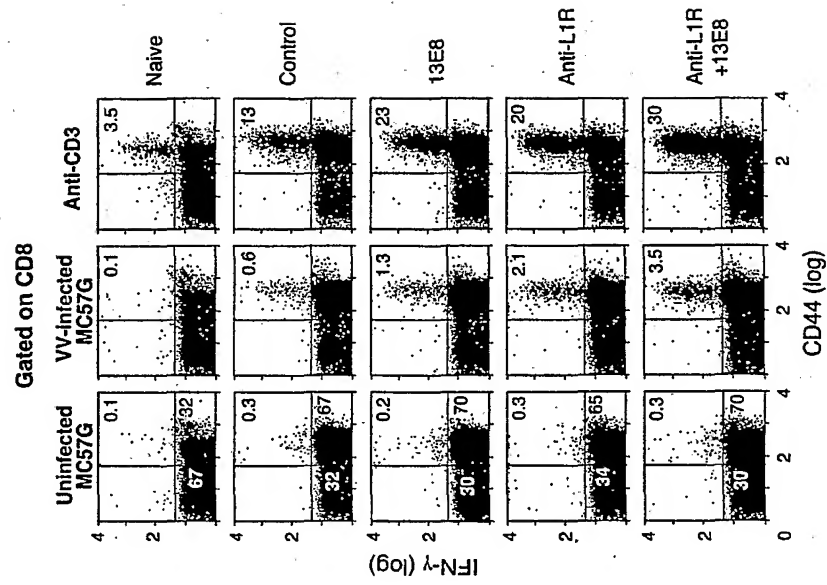


Fig. 17

Fig. 8A

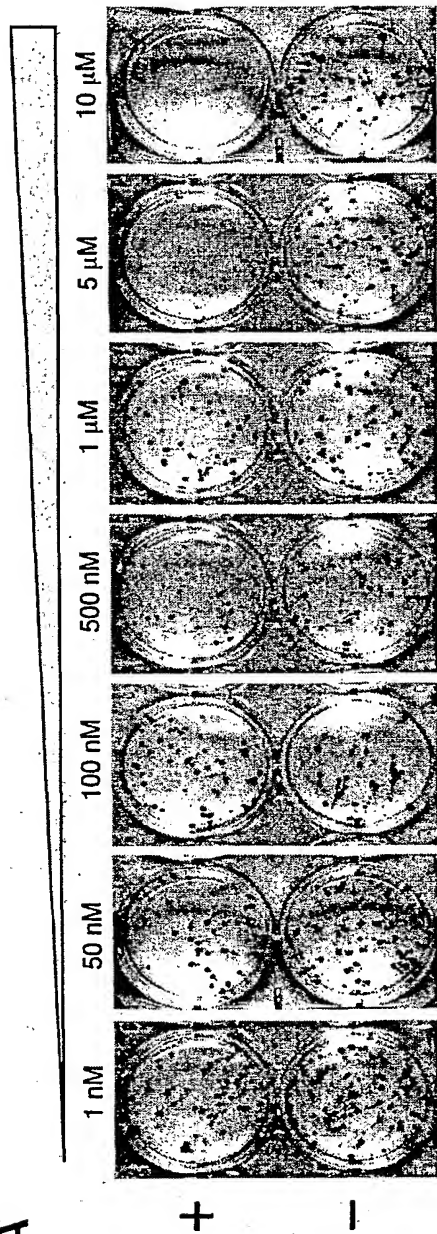


Fig. 8B

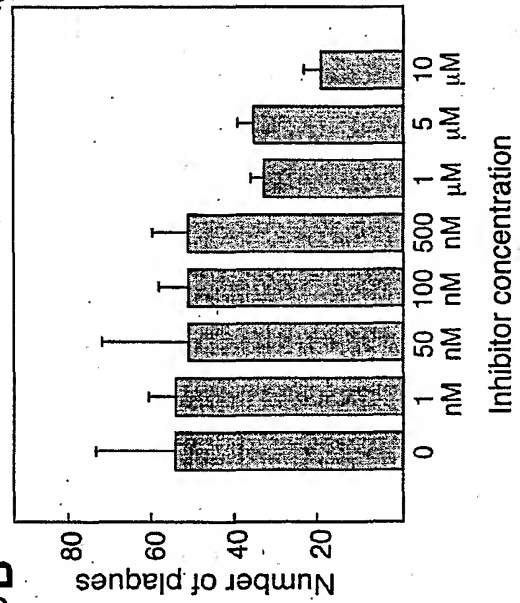
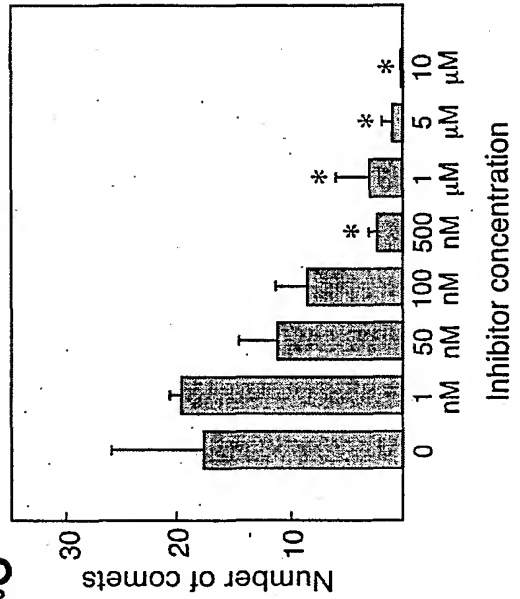


Fig. 8C



(19) World Intellectual Property Organization  
International Bureau



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13 January 2005 (13.01.2005)

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

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**Published:**

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(88) Date of publication of the international search report:  
23 March 2006

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: INHIBITION OF VIRAL PATHOGENESIS

(57) Abstract: The invention features methods of inhibiting activation of cells by viral erb-B ligands and methods of enhancing immune responses in animals (e.g., human subjects) infected, that will be infected, or are at risk of being infected with a virus whose genome contains a nucleic acid sequence encoding a viral erb-B ligand.



WO 2005/003325 A3

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/07537

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) : A61K 39/395

US CL : 424/143.1, 172.1, 174.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/143.1, 172.1, 174.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| X          | WO 01/21192 A2 (CORIXA CORPORATION) 29 March 2001 (29.03.2001), claim 27, page 2 lines 23-28, page 31 lines 7-8.   | 1, 2, 13, 28          |
| A,T        | WANG et al. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature. 24 July 2003, Vol. 424, pages 456-461.            | 1, 2, 13, 28          |
| A          | TZAHAR et al. Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network. EMBO Journal, 1998, Vol. 17, No. 20, pages 5948-5963. | 1-13, 28-43           |

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

30 December 2005 (30.12.2005)

Date of mailing of the international search report

31 JAN 2006

Name and mailing address of the ISA/US

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Authorized officer

Mary E. Mosher, Ph.D.

Telephone No. 571-272-1600

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/07537

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-13 and 28-43

- Remark on Protest
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
  - ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
  - ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US04/07537

### BOX III. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Search Authority has found 13 inventions claimed in the International Application covered by the claims indicated below.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

\*Group I, claim(s) 1-43, drawn to a method of treatment whereby an animal is treated with a compound that inhibits the activity of an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase.

\*Note that if Applicant elects to have additional inventions search from Group I Applicant must further elect from the subgroups of Group I (listed below).

Group II, claim(s) 45 and 46, drawn to an in vitro method whereby an isolated compound that binds to an erb-B tyrosine kinase and inhibits the activity of the tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase receptor is contacted with a cell that expresses the erb-B tyrosine kinase along with a temporally-related contact of the cell with a viral erb-B ligand or fragment said ligand.

Group III, claim(s) 44 and 47, in pad, drawn to a method of treatment whereby an animal is treated with (1) an antibody that substantially neutralizes one or more forms of the virus and (2) a compound that inhibits the activity of an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase.

Group IV, claim(s) 44 and 47, in pad, drawn to a method of treatment whereby an animal is treated with (1) a vaccine that stimulates an immune response against the virus and (2) a compound that inhibits the activity of an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase.

Group V, claim(s) 48, drawn to a method of determining whether a compound is an antiviral compound via administration of a compound that inhibits erb-B tyrosine kinase activity to a virus-susceptible animal along with a temporally-related challenge of the animal with said virus.

Group VI, claim(s) 49-61, in part, drawn to a method of treating an animal with (1) an antibody that binds to an erb-B ligand and (2) and antibody that substantially neutralizes one or more forms of the virus.

Group VII, claim(s) 49-61, in pad, drawn to a method of treating an animal with (1) an antibody that binds to an erb-B ligand and (2) and a vaccine that stimulates an immune response against the virus.

#### Subgroups of Group I:

Subgroup IA, claim(s) 1, in part, and 13, drawn to the method where the compound is a nonagonist antibody that binds to the erb-B tyrosine kinase.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US04/07537

Subgroup IB, claim(s) 1, in part, and 14, drawn to the method where the compound is a nonagonist erb-B ligand or a fragment of said ligand.

Subgroup IC, claim(s) 1 and 15, in part, and 16, 20, 23 and 24, drawn to the method where the compound is a quinazoline-based compound.

Subgroup ID, claim(s) 1 and 15, in part, and 17 and 25, drawn to the method where the compound is a pyridopyrimidine-based compound.

Subgroup IE, claim(s) 1 and 15, in part, and 18 and 26, drawn to the method where the compound is a quinoline-3-carbonitrile-based compound.

Subgroup IF, claim(s) 1 and 15, in part, and 19 and 27, drawn to the method where the compound is a pyrrolpyrimidine-based compound.

Subgroup IG, claim(s) 1 and 15, in part, and 21 and 22, drawn to the method where the compound is an irreversible inhibitor of the erb-B tyrosine kinase activity.

### As to claims 2-12 and 28-43:

The technical feature of claim 1 is the compound that inhibits the activity of an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase used in the method. As explained below, this technical feature does not constitute a special technical feature as it does not make a contribution over the prior art. Thus, the "special technical feature" is presumed by the Examiner to relate to the identity of the compound itself. Subgroups IA through IG are divided on the basis of the identity of the compound. Other claims, such as claims 38 through 42, specify the type of immunological reaction produced. It is not possible for the Examiner to determine a priori which of the compounds will produce which type of response. Moreover, it is possible that a certain response will be applicable to some but not all compounds, yet the division of all responses would not necessarily be coextensive with the division of the classes. A similar issue can be raised about the other claims of Group I not included in the above subgroupings, as these dependent claims address qualitative features unrelated to the identity of the compound. Applicant can direct the Examiner as to which of claims 2-12 and 28-43 apply to which subgroups listed above.

I. This International Searching Authority considers that the international application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I-VII do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

According to PCT Rule 13.2, unity of invention exists only when there is a shared, same or corresponding special technical feature among the claimed inventions. Furthermore, according to PCT Rule 13.2, unity of invention exists only when the shared, same or corresponding special technical feature is a contribution over the prior art.

The technical feature linking Groups I-V is that they all relate to a compound that inhibits the activity of an erb-B tyrosine kinase or inhibits the activation of an erb-B tyrosine kinase in the context of a virus containing a gene encoding a viral erb-B ligand, wherein the compound is further used in a method.

Groups VI and VII do not share this technical feature. Rather, the claims of Groups VI and VII are directed at antibodies that bind the viral erb-B ligand. This would not necessarily inhibit the activity or activation of the erb-B tyrosine kinase as the receptor also binds I cellular ligands.

As to Groups I-V, the technical feature is not a "special technical feature" as it does not make a contribution over the prior art. Compounds that inhibit the activation or activity of an erb-B tyrosine kinase by the viral erb-B ligand are taught by Eppstein et al. (1985) Nature 318:663-665. Epstein teaches that fragments of the vaccinia virus peptide VGF, a viral erb-B ligand, inhibited vaccinia virus infection by occupying the EGF receptor, an erb-B tyrosine kinase. In the process of binding the receptor, Epstein's peptide fragments would thereby inhibit the activation of the erb-B tyrosine kinase by VGF. Thus, Epstein's teachings shows the claims directed at the genus of compounds that inhibit the activation of an erb-B tyrosine kinase by the viral erb-B ligand lack novelty or inventive step, and thus do not make a contribution over the prior art.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/07537

Continuation of B. FIELDS SEARCHED Item 3:

EAST USPat, PGPub, EPO, JPO, Derwent; Dialog files 155, 357. Search terms: ERB(W)(B OR B1 OR B2 OR B3 OR B4), ERB(W)B?, ERBB?, EGFR, EGF(W)RECEPTOR?, EGF(2N)RECEPTOR?, EGF?, HER1, HER2, HER ADJ "2" NEU, TYROSINE(W)KINASE, VACCINIA, VARIOLA, SMALLPOX, MONKEYPOX, POX?, \$POX, POX\$, ORTHOPOX?, VIRUS, VIRAL, ANTIVIR?, INFECT?, ANTI, ANTIBOD?, LIGAND, ANALOG, MONOCLONAL, VIVO, TRASTUZMAB, TRASTUZUMAB, HERCEPTIN, THERAP?, PREVENT\$, TREAT\$, PY<2004, HUMAN, HEPATITIS ADJ B, HBV, EPSTEIN ADJ BARR, EBV, CYTOMEGALO?, CMV, REOVIR\$, VIRULENCE, RECEPTOR?